

### ***Remarks***

Reconsideration of this Application is respectfully requested.

Claims 10-22, 47 and 56-75 are pending in the application, with claims 10-12, 18, 20, 47 and 68-73 being the independent claims.

Based on the following remarks, Applicants respectfully request that the Examiner reconsider all outstanding rejections and that they be withdrawn.

#### ***I. Claim Rejections Under 35 U.S.C. § 112, First Paragraph***

Claims 10-22, 47 and 56-67 were rejected under 35 U.S.C. § 112, first paragraph, as allegedly containing subject matter which was not described in the specification in such a way as to reasonably convey to one of ordinary skill in the art that the inventors, at the time the application was filed, had possession of the claimed invention. (*See* Paper No. 27, page 2.) According to the Examiner, "the new limitation of 'with the proviso that said one or more detectably labeled oligonucleotides do not comprise an acceptor molecule' in each of the independent claims appears to represent new matter." (*See id.*) Applicants respectfully disagree with this assessment and traverse this rejection.

A negative proviso in a claim does not violate 35 U.S.C. § 112, first paragraph, if Applicants have conveyed with reasonable clarity to those skilled in the art that, as of the effective filing date, Applicants were in possession of the invention. *See Vas-Cath, Inc. v. Mahurkar*, 935 F.2d 1555, 1560, 19 USPQ2d 1111, 1117 (Fed. Cir. 1991); *see also In re Wright*, 866 F.2d 422, 9 USPQ2d 1649 (Fed. Cir. 1989). Here, Applicants have conveyed

to those of ordinary skill in the art that, as of the effective filing date, Applicants were in possession of methods of quantification, detection, and amplification of nucleic acid molecules involving the use of detectably labeled oligonucleotides that do not comprise an acceptor molecule. Therefore, the language at issue does not constitute new matter.

The present invention is an advance over previously known methods of quantification, detection, and amplification of nucleic acid molecules. For example, according to at least certain aspects of the present invention, a label attached to an oligonucleotide will produce a detectable change in an observable property (*e.g.*, fluorescence intensity) upon becoming part of a double stranded molecule *without a second molecule* -- an acceptor molecule -- to accept the energy emitted from the label. As stated in the specification, "[t]he method does not require the presence of any specific quenching moiety or detection oligonucleotide." (*See* specification at page 70, lines 13-15.) This aspect of the invention is illustrated explicitly in several of the examples.

Example 4, for instance, involves the hybridization of "oligo A," an oligonucleotide that is internally labeled with only a *single* fluorescein, to unlabeled, complementary "oligo C." (*See* specification at page 70, line 28 through page 71, line 3.) There is no acceptor molecule on oligo A or on oligo C. (*See* specification at page 66, Table 2.) Surprisingly, however, the fluorescence signal was shown to increase when oligo A became part of a double stranded molecule with oligo C. (*See* specification at page 71, lines 5-8, and Fig. 2.)

A similar result was obtained in Example 7. (*See* specification at page 73, lines 5-21.) Here, the same singly labeled oligo A as in Example 4 was used as a reverse primer for IL4 cDNA amplification. The polymerase chain reaction was performed using oligo A, unlabeled oligo I (IL4 forward primer), and varying amounts of IL4 template. (*See*

specification at page 73, lines 12-17.) The results demonstrate a detectable change in fluorescence as the PCRs progressed even though there was no acceptor molecule included in any of the reagents. (See specification at page 73, lines 17-18, and Fig. 5.) As stated in the specification, "[t]he results of this experiment demonstrate that although no quencher is present in the structure of labeled oligonucleotide, it can be successfully used in quantitative PCR." (See specification at page 73, lines 19-21.) Other experiments involving the use of detectably labeled oligonucleotides that were designed specifically *not* to comprise an acceptor molecule are illustrated in Examples 8, 9 and 10.

There are other places throughout the specification that demonstrate possession of the claimed methods of quantification, detection and amplification of nucleic acid molecules involving detectably labeled oligonucleotides that do not comprise an acceptor molecule. In describing how the present invention is an improvement over previously known methods, for example, it is stated that the compounds and methods of the invention "do not require labeling of oligonucleotides with two different compounds (*like FRET-based methods*), and thus simplify the production of the labeled oligonucleotides." (See specification at page 10, lines 17-20, emphasis added.) Fluorescence resonance energy transfer ("FRET")-based methods of nucleic acid detection and quantitaion necessarily involve the use of an oligonucleotide that contains an energy-emitting moiety (donor) *and* an energy acceptor. (See, *e.g.*, specification at page 3, lines 3-6.) Thus, the distinction over FRET-based methods recited in the specification would indicate to a skilled artisan that, in at least certain embodiments of the invention, oligonucleotides are used that do not comprise an acceptor molecule.

The Examiner has cited two passages from the specification to support this rejection. First, the Examiner cited a passage located in the specification at page 44, lines 4-6 ("... the label is any moiety which undergoes a detectable change in any observable property upon hybridization . . .") This passage does not suggest the presence of or need for an acceptor molecule. As discussed above, one of the surprising and unexpected findings by the inventors is that a labeled oligonucleotide can undergo a detectable change in an observable property upon becoming part of a double stranded molecule *without the presence of an acceptor molecule*.

The second passage cited by the Examiner is at page 24, lines 15-16 ("In another embodiment of the invention, the label is a member of a FRET pair.") This passage, however, only refers to one particular embodiment of the invention. In other embodiments described in the specification an acceptor molecule is intentionally excluded from the detectably labeled oligonucleotides. (*See discussion above.*)

A person of ordinary skill in the art would appreciate that Applicants were in possession of methods of quantification, detection and amplification of nucleic acid molecules using detectably labeled oligonucleotides that do not comprise an acceptor molecule. Therefore, Applicants respectfully request that the rejection of claims 10-22, 47 and 56-67 under 35 U.S.C. § 112, first paragraph, be reconsidered and withdrawn.

## ***II. Claim Rejections Under 35 U.S.C. § 112, Second Paragraph***

Claims 10-22, 47 and 56-67 were rejected under 35 U.S.C. § 112, second paragraph, as allegedly being indefinite for failing to particularly point out and distinctly claim the

subject matter which Applicants regard as the invention. (*See* Paper No. 27, page 4.)

Applicants respectfully traverse this rejection.

This rejection is directed to the term "acceptor" recited in the claims. The Examiner stated that "there is no definition of this term in the specification and no mention of the term was even found in the specification. It is unclear what the scope of the term 'acceptor' is with regard to the claim." (*See* Paper No. 27, page 4.) The Examiner has interpreted "acceptor" to mean an acceptor that re-emits a signal. (*See* Paper No. 27, page 4 and page 7.) According to the Examiner, "the term 'acceptor' is interpreted to be limited to the sort of acceptors used in Heller, which reemit the fluorescence energy for detection at a different wavelength." (*See* Paper No. 27, page 7.) Applicants respectfully disagree with the Examiner's interpretation of "acceptor" and assert that this term does not render the claims indefinite.

A rejection under 35 U.S.C. §112, second paragraph, is appropriate only if the scope of the invention sought to be patented cannot be determined from the language of the claims with a reasonable degree of certainty. *See In re Wiggins*, 488 F.2d 538, 179 USPQ 421 (CCPA 1973). Definiteness of claim language must be analyzed, not in a vacuum, but in light of: (a) the content of the application disclosure; (b) the teachings in the art; and (c) the claim interpretation that would be given by one having ordinary skill in the art. *See* MPEP § 2173.02. In view of these factors, a person of ordinary skill in the art would understand the scope of the subject matter encompassed by the claims that include the term "acceptor." More specifically, an "acceptor" would have been understood to mean a molecule to which energy is transferred from an energy-emitting donor moiety. "Acceptor" would not be interpreted to mean only a molecule that re-emits fluorescent energy, but would be

understood to also encompass molecules that accept but do not emit energy; *i.e.*, quenchers. (See discussion below.) Therefore, the rejection under § 112, second paragraph was improper.

**A. The Content of the Application Disclosure**

The term "acceptor" is found in several places throughout the specification. For example, in describing nucleic acid detection methods that involve FRET, it is stated that "[t]hese methods utilize the phenomena of fluorescence resonance energy transfer (FRET) in which the energy from an excited fluorescent moiety is transferred to an *acceptor* molecule when the two molecules are in close proximity to each other." (Specification at page 3, lines 3-6, emphasis added.) Moreover, in describing the method of Wang *et al.*, *Anal. Chem.* 67:1197-1203 (1995), the specification notes that:

The Wang *et al.* method uses an "energy sink" oligonucleotide complementary to the reverse primer. The "energy sink" and reverse-primer oligonucleotide have donor and *acceptor* labels, respectively. Prior to amplification, the labeled oligonucleotides form a primer duplex in which energy transfer occurs freely.

(Specification at page 8, lines 10-14, emphasis added.) From these passages alone, a person of ordinary skill in the art would understand the term "acceptor" to mean a molecule to which energy is transferred from an energy-emitting donor moiety.

The specification also makes it clear that the term "acceptor" includes quenchers. In describing the system of Tyagi and Kramer (*Nature Biotech.* 14:303-309 (1996)), the specification states: "In the case of the Tyagi and Kramer method, this acceptor moiety is a quencher, that is, the acceptor absorbs energy released by the donor, but then does not itself fluoresce." (Specification at page 9, lines 9-11.) In view of this passage, a person of

ordinary skill in the art would understand the meaning of the term "acceptor" and would appreciate that a quencher is an acceptor.

***B. The Teachings in the Art and The Interpretation Given By Those of Ordinary Skill In the Art***

Persons of ordinary skill in the relevant art are very familiar with the term "acceptor" and the types of molecules that qualify as acceptors. (*See, e.g., Selvin, Methods Enzymol.* 246:300-334 (1995) ("Selvin"), copy submitted herewith as Exhibit 1.) According to Selvin, "[i]n FRET, a fluorescent donor molecule transfers energy to an *acceptor molecule*, which is usually but not necessarily a fluorescent molecule. The mechanism is a nonradiative induced dipole-induced dipole interaction." (Selvin at page 306, internal citation omitted.) Thus, it is clear that an acceptor is a molecule to which energy is transferred from a donor.

The term "acceptor" is understood by persons of ordinary skill in the art as including quenchers. As stated in Nazarenko *et al., Nucleic Acids Research* 25:2516-2521 (1997) ("Nazarenko"): "FRET is a process in which the energy from an excited fluorophore is transferred to an acceptor moiety at distances up to 70-100 Å. As a result, the emission of the fluorophore is quenched." (Nazarenko at page 2516, right column, internal citation omitted.) Nazarenko also states that "[i]n our method, donor and acceptor (quencher) moieties are both attached to a hairpin structure on the 5' end of the amplification primer." (Nazarenko at page 2517, left column.) Nazarenko uses the terms "acceptor" and "quencher" interchangeably, indicating that quenchers are acceptors.

The Examiner has relied on Heller (U.S. Patent No. 5,565,322) to support the assertion that an "acceptor" should be limited to mean acceptors that re-emit a signal. (*See* Paper No. 27, page 7.) Although Heller refers to acceptors that re-emit the energy

transferred from a donor, the term "acceptor" would not be interpreted as being limited to these kinds of acceptors. A person of ordinary skill in the art would appreciate that, depending on the attributes of the acceptor and the wavelength of energy emitted from the donor, an acceptor can either accept the energy and re-emit it at a different wavelength (as in Heller) or simply absorb the energy from the donor and quench it (as in Nazarenko). (*See, e.g.,* Wu and Brand, *Analytical Biochem.* 218:1-13 (1994) ("Wu and Brand"), copy submitted herewith as Exhibit 2.)

*Donor quenching.* This is probably the most commonly used method. Excitation is set at the wavelength of donor absorption and the emission of donor is monitored. The emission wavelength of donor is selected such that no contribution from acceptor fluorescence is observed. The presence of acceptor quenches donor fluorescence.

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*Acceptor enhancement.* If an acceptor is fluorescent its fluorescence intensity is enhanced when energy transfer occurs (with excitation into the donor). This is another means to visualize energy transfer from a fluorescence spectrum. In an emission spectrum, one excites at the wavelength of donor absorption and observes the intensity increase of acceptor.

(Wu and Brand at page 5, right column and at page 6, left column.)

### **C. Summary**

Based on the disclosure in the specification and the understanding of those of ordinary skill in the art, the meaning of the term "acceptor" would be clearly understood. This term does not render the claims indefinite. Applicants therefore respectfully request that the rejection of claims 10-22, 47 and 56-67 under 35 U.S.C. § 112, second paragraph, be reconsidered and withdrawn.



### **III. Claim Rejections Under 35 U.S.C. § 102**

#### **A. Livak**

Claims 10-17, 47, 56-58, 62, 64, 66, 67-70, 72 and 73 were rejected under 35 U.S.C. § 102(b) as allegedly being anticipated by Livak *et al.*, WO 96/15270 ("Livak"). (See Paper No. 27, page 5.) Applicants respectfully traverse this rejection.

An anticipation rejection under 35 USC § 102 requires a showing that each limitation of a claim is found in a single reference, practice, or device. *See In re Donohue*, 766 F.2d 531, 226 USPQ 619, 621 (Fed. Cir. 1985). Since not every element of claims 10-17, 47, 56-58, 62, 64, 66, 67-70, 72 and 73 is found in Livak, the rejection under § 102 was improper and should be withdrawn.

#### **1. Claims 10-17, 47 56-58, 62, 64, 66 and 67**

Claims 10-17, 47, 56-58, 62, 64, 66 and 67 are directed to methods involving the use of one or more detectably labeled oligonucleotides that:

- (a) do not comprise an acceptor molecule; and
- (b) comprise one or more detectable labels located *only* internally.

The oligonucleotides of Livak all comprise a quencher molecule. (See Livak at page 10, lines 16-18.) A quencher is an acceptor. (See discussion at Section II, above.) In addition, the oligonucleotides of Livak all comprise a detectable label located at the 5' end. (See Livak at page 21, lines 17-19, and at page 22, Table 1.) That is, the oligonucleotides of Livak do not comprise one or more detectable labels located *only* internally.

Since the oligonucleotides of Livak: (a) comprise an acceptor molecule, and (b) comprise a detectable label on the 5' end (*i.e.*, do not comprise one or more detectable labels

located *only* internally), the methods of Livak cannot and do not anticipate claims 10-17, 47, 64, 66 and 67.

## **2. Claims 68-70, 72 and 73**

Claims 68-70, 72 and 73 are directed to methods involving the use of one or more detectably labeled oligonucleotides that:

- (a) comprise one or more detectable labels located *only* internally; and
- (b) are hairpin oligonucleotides.

The oligonucleotides of Livak all comprise a detectable label located at the 5' end. (See Livak at page 21, lines 17-19, and at page 22, Table 1.) That is, the oligonucleotides of Livak do not comprise one or more detectable labels located *only* internally. In addition, the oligonucleotides of Livak are not hairpin oligonucleotides. (See Livak at page 22, Table 1.)

The Examiner stated that "Livak teaches the use of hairpin probes." (Paper No. 27, page 6.) To support this assertion, the Examiner cited Livak at page 2. The section of Livak cited by the examiner, however, discusses hairpin probes that have been described *elsewhere* and have been used in methods distinct from those described in Livak. (See Livak at page 2, lines 4-17.) Furthermore, Livak discusses the disadvantages of hairpin probes. (See Livak at page 2, lines 15-17.) Thus, the methods of Livak do not involve the use of oligonucleotides having a hairpin structure.

Since the oligonucleotides of Livak: (a) comprise a detectable label on the 5' end (*i.e.*, do not comprise one or more detectable labels located *only* internally), and (b) are not hairpin oligonucleotides, the methods of Livak cannot and do not anticipate claims 68-70, 72 and 73.

**B. Nazarenko**

Claims 18-22, 59-61, 66, 67 and 71 were rejected under 35 U.S.C. § 102(b) as allegedly being anticipated by Nazarenko *et al.*, *Nuc. Acids Res.* 25:2516-2521 (1997) ("Nazarenko"). (*See* Paper No. 27, page 6.) Applicants respectfully traverse this rejection. Since not every element of claims 18-22, 59-61, 66, 67 and 71 is found in Nazarenko, the rejection under § 102 was improper and should be withdrawn.

**1. Claims 18-22, 59-61, 66 and 67**

Claims 18-22, 59-61, 66 and 67 are directed to methods involving the use of one or more detectably labeled oligonucleotides that:

- (a) do not comprise an acceptor molecule; and
- (b) comprise one or more detectable labels located *only* internally.

The oligonucleotides of Nazarenko all comprise an acceptor molecule. (*See, e.g.*, Nazarenko abstract: "The PCR primers contain hairpin structures on their 5' ends with donor and *acceptor* moieties located in close proximity on the hairpin stem." Emphasis added.) In addition, the oligonucleotides of Nazarenko all comprise a detectable label located at the 5' end. (*See* Nazarenko at page 2517, right column and Table 1, and at page 2518, left column.) That is, the oligonucleotides of Nazarenko do not comprise one or more detectable labels located *only* internally.

Since the oligonucleotides of Nazarenko: (a) comprise an acceptor molecule, and (b) comprise a detectable label on the 5' end (*i.e.*, do not comprise one or more detectable labels located *only* internally), the method of Nazarenko cannot and does not anticipate claims 18-22, 59-61, 66 and 67.

**2. Claim 71**

Claim 71 is directed to methods involving the use of one or more detectably labeled oligonucleotides that comprise one or more detectable labels located *only* internally. As discussed above, the oligonucleotides of Nazarenko do not comprise one or more detectable labels located *only* internally. Therefore the method of Nazarenko cannot and does not anticipate claim 71.

**IV. Claim Rejections Under 35 U.S.C. § 103**

**A. Heller In View of Nazarenko**

Claims 68-75 were rejected under 35 U.S.C. § 103(a) as allegedly being unpatentable over Heller, U.S. Patent No. 5,565,322 ("Heller") in view of Nazarenko. (*See* Paper No. 27, page 7.) Applicants respectfully traverse this rejection.

In order to establish a *prima facie* case of obviousness, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the references or to combine reference teachings. *See In re Rouffet*, 149 F.3d 1350, 1357, 47 USPQ2d 1453, 1457-58 (Fed. Cir. 1998). Since there is nothing in either Heller or Nazarenko to suggest combining or modifying the disclosures, a *prima facie* case of obviousness has not been established.

The Examiner asserted that "an ordinary practitioner using the Heller system is expressly motivated, in diagnostic applications, to reduce background using the Heller methodology and would be motivated to reduce background to as low a level as possible."

(Paper No. 27, page 9.) To support this assertion, the Examiner cited the following passage from Heller:

A multiple donor system comprised of such non-fluorescent chromophores would have very little inherent fluorescent background. This property overcomes a major limitation that has severely limited practical uses of fluorescent energy transfer in DNA diagnostic assay applications.

(Heller at column 10, lines 23-27.) This passage, however, does not suggest a need to modify the Heller method to reduce background fluorescence. To the contrary, this passage indicates that the Heller method itself has overcome the problem of inherent fluorescent background. This fact is made even more clear when one considers the paragraph immediately preceding the passage cited by the Examiner:

A non-fluorescent donor producing fluorescent re-emission in the acceptor group is an extremely valuable property. The non-fluorescing donor in a composition of the present invention *provides the particular advantage of a low or absent level of emission by the donor*, thereby not contributing to background or the detectable emitted light in a donor-acceptor system. Thus, non-fluorescent donors allow for very low background and are particularly preferred.

(Heller at column 10, lines 15-22, emphasis added.) Since Heller indicates that the method set forth therein solves the problem of background fluorescence, a person of ordinary skill in the art would have no motivation to modify the Heller method to "reduce background."

Moreover, the system described in Heller is designed to transfer photonic energy over long distances by relaying the energy between multiple donors to an acceptor.

It has now been discovered that multiple chromophore donor groups which are located beyond the normal Förster distance ( $>5$  nm) can be arranged to absorb and transfer photonic energy to a terminal acceptor group thereby acting as a light antenna or photonic conductor.

(Heller at column 4, lines 28-32; *see also* Figs. 1B and 3B.) Therefore, introducing a hairpin structure into the Heller oligonucleotides would appear to defeat the ultimate purpose of the invention. That is, if the oligonucleotides were in a hairpin conformation, the signal would no longer be relayed between multiple donors to an acceptor over a long distance.

In sum, Heller indicates that its system overcomes the problem of background fluorescence. The Examiner has not pointed to anything in Heller suggesting that the method set forth therein could or should be improved by "reduc[ing] background to as low a level as possible." In addition, the purpose of the Heller system would be defeated by including hairpin structures into the oligonucleotides set forth therein. Therefore, a person of ordinary skill in the art would not be motivated to modify the disclosure of Heller or combine it with that of Nazarenko. Applicants respectfully request that the rejection of claims 68-75 under 35 U.S.C. § 103(a) be reconsidered and withdrawn.

***B. Nazarenko***

Claims 18-22, 59-61, 63-67 and 71 were rejected under 35 U.S.C. § 103(a) as allegedly being unpatentable over Nazarenko. (*See* Paper No. 27, page 10.) Applicants respectfully traverse this rejection.

In order to establish a *prima facie* case of obviousness, all the claim limitations must be taught or suggested by the prior art. *See In re Royka*, 490 F.2d 981, 180 USPQ 580 (CCPA 1974). Since Nazarenko does not teach or suggest all of the elements of the claims, a *prima facie* case of obviousness cannot be established.

As discussed above, the claims in the present application are directed to methods involving the use of one or more detectably labeled oligonucleotides that:

- (a) do not comprise an acceptor molecule; and/or

(b) comprise one or more detectable labels located *only* internally.

The oligonucleotides of Nazarenko: (a) comprise an acceptor molecule, and (b) comprise a detectable label on the 5' end (*i.e.*, do not comprise one or more detectable labels located *only* internally). Therefore, Nazarenko does not teach or suggest all of the elements of the present claims. Accordingly, a *prima facie* case of obviousness cannot be established. Applicants therefore respectfully request that the rejection of claims 18-22, 59-61, 63-67 and 71 under 35 U.S.C. § 103(a) be reconsidered and withdrawn.

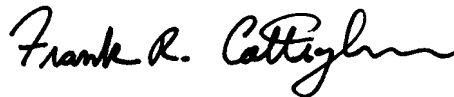
### ***Conclusion***

All of the stated grounds of rejection have been properly traversed, accommodated, or rendered moot. Applicants therefore respectfully request that the Examiner reconsider all presently outstanding rejections and that they be withdrawn. Applicants believe that a full and complete reply has been made to the outstanding Office Action and, as such, the present application is in condition for allowance. If the Examiner believes, for any reason, that personal communication will expedite prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided.

Prompt and favorable consideration of this Amendment and Reply is respectfully requested.

Respectfully submitted,

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of the extent of binding. This effect (known informally as "Chip dip") is illustrated in Figs. 6 and 7 which concern the ethidium bromide/tRNA system.<sup>41</sup> In this case the free ethidium bromide has a lifetime of 1.86 nsec and a rotational relaxation time of 0.54 nsec. On binding the Phe-tRNA<sup>Phe</sup> the lifetime increases to 26 nsec and the rotational relaxation increases to 136 nsec. A single rotating species cannot give rise to a negative value for the differential phase delay (assuming excitation at a wavelength which gives a positive  $P_0$ ), and the negative excursion for this function shown in Fig. 6 is due to the presence of free ethidium bromide in equilibrium with bound material. Figure 7 shows the calculated effects for increasing amounts of bound material.

We should note that, given the difference in quantum yield between the free and bound probe, the fractional intensities utilized in Fig. 7 actually represent small percentages of bound probe on a molar basis. In fact, considering the accuracy of the differential phase measurement (better than 0.1°) one can detect, in this system, on the order of 0.1% bound probe. This phenomenon also occurs in time-domain measurements. Specifically, if one monitors the anisotropy decay of a system which displays multiple lifetimes associated with multiple rotational diffusion rates then one may observe a decline at short times of the anisotropy followed by a rise at latter times and subsequent decrease. This "dip and rise" effect has been observed by Millar and co-workers<sup>22</sup> in studies on protein-DNA interactions, specifically in the case of the interaction of a fluorescent DNA duplex with the Klenow fragment of DNA polymerase.

#### Acknowledgments

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### [13] Fluorescence Resonance Energy Transfer

By PAUL R. SELVIN

#### Introduction

Fluorescence resonance energy transfer (FRET) is a technique for measuring the distance between two points which are separated by approximately 10–75 Å. The technique is valuable because measurements can be made under physiological (or other) conditions with near angstrom

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resolution and with the exquisite sensitivity of fluorescence measurements. For these reasons FRET has found wide use in polymer science, biochemistry, and structural biology. Reviews have appeared on FRET applied to actin structure,<sup>1</sup> nucleic acids,<sup>2</sup> phycobiliproteins,<sup>3-5</sup> cell surfaces with an emphasis on protein interaction,<sup>6</sup> *in situ* imaging,<sup>7</sup> diffusion,<sup>8</sup> and microscopy.<sup>9-12</sup> Among systems already studied in the literature are DNAs such as oligonucleotides<sup>13-16</sup> and Holliday junctions<sup>14,17,18</sup>; proteins such as oligopeptides,<sup>19</sup> rhodopsin,<sup>8,20</sup> myosin,<sup>21</sup> various calcium binders,<sup>22</sup> and major histocompatibility complexes<sup>23</sup>; RNA<sup>24,25</sup>; and nucleic

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<sup>2</sup> R. M. Clegg, this series, Vol. 211, p. 353.

<sup>3</sup> A. N. Glazer and L. Stryer, this series, Vol. 184, p. 188.

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<sup>15</sup> H. Ozaki and L. W. McLaughlin, *Nucleic Acids Res.* **20**, 5205 (1992).

<sup>16</sup> R. M. Clegg, A. I. Murchie, A. Zechel, and D. M. Lilley, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 2994 (1993).

<sup>17</sup> A. I. Murchie, R. M. Clegg, E. von Kitzing, D. R. Duckett, S. Diekmann, and D. M. Lilley, *Nature (London)* **341**, 763 (1989).

<sup>18</sup> R. M. Clegg, A. I. H. Murchie, A. Zechel, C. Carlberg, S. Diekmann, and D. M. J. Lilley, *Biochemistry* **31**, 4846 (1992).

<sup>19</sup> L. Stryer and R. P. Haugland, *Proc. Natl. Acad. Sci. U.S.A.* **98**, 719 (1967).

<sup>20</sup> R. O. Leder, S. L. Helgerson, and D. D. Thomas, *J. Mol. Biol.* **209**, 683 (1989).

<sup>21</sup> H. C. Cheung, I. Gryczynski, H. Malak, G. Wicz, M. L. Johnson, and J. R. Lakowicz, *Biophys. Chem.* **40**, 1 (1991).

<sup>22</sup> D. T. Counce and W. D. Horrocks, Jr., *Biochemistry* **31**, 7963 (1992).

<sup>23</sup> R. Tampé, B. R. Clark, and H. M. McConnell, *Science* **254**, 87 (1991).

<sup>24</sup> D. J. Robbins, O. W. J. Odom, J. Lynch, D. Dottavio-Martin, G. Kramer, and B. Hardesty, *Biochemistry* **20**, 5301 (1981).

<sup>25</sup> K. Beardsley and C. R. Cantor, *Proc. Natl. Acad. Sci. U.S.A.* **65**, 39 (1970).

acid-protein complexes such as nucleosomes,<sup>26-29</sup> chromatin<sup>30,31</sup> and protein-promoter interactions.<sup>32</sup> FRET has also been used to monitor dynamic processes such as actin assembly,<sup>1,33</sup> nucleosome assembly, and human immunodeficiency virus (HIV) protease activity.<sup>34</sup> Van der Meer and co-workers have recently written a book on FRET.<sup>35</sup> Excellent reviews of the basics of FRET, which complement this chapter, have appeared.<sup>36-38</sup>

The idea behind the technique is to label the two points of interest with different dyes; one, which must be fluorescent, is called the donor, and the other, which is not necessarily fluorescent but often is, is called the acceptor. By choosing dyes with the appropriate spectral characteristics, the donor, after being excited by light, can transfer energy to the acceptor. The efficiency of energy transfer depends on the inverse sixth power of the distance between the dyes. In general, the acceptor must be within 10–75 Å to get reasonable energy transfer, the exact range depending on the dyes chosen.

If one measures the amount of energy transfer, it is therefore possible to determine the distance between donor and acceptor. Qualitatively, the farther apart the donor and acceptor, the less energy transfer. The extent of energy transfer can be measured because the fluorescence of the donor (both intensity and lifetime) decreases, or is quenched, and the acceptor, if fluorescent, increases its fluorescence, or becomes "sensitized," with energy transfer. These changes in fluorescence can be measured by comparing a complex labeled with both donor and acceptor to ones labeled only with donor and only with acceptor. The experimental and theoretical details are presented below.

### Other Uses

Measuring the (static) distance between two points, although the main use of FRET, is just one application. A number of workers have also used

<sup>26</sup> H. Eshaghpour, A. E. Dieterich, D. Crothers, and C. R. Cantor, *Biochemistry* **19**, 1797 (1980).

<sup>27</sup> D. G. Chung and P. N. Lewis, *Biochemistry* **25**, 5036 (1986).

<sup>28</sup> D. G. Chung and P. N. Lewis, *Biochemistry* **25**, 2048 (1986).

<sup>29</sup> J. Widom, in preparation (1994).

<sup>30</sup> M. Lerho, M. Favazza, and C. Houssier, *J. Biomol. Struct. Dyn.* **7**, 1301 (1990).

<sup>31</sup> G. Sarlet, S. Muller, and C. Houssier, *J. Biomol. Struct. Dyn.* **10**, 35 (1992).

<sup>32</sup> T. Heyduk and J. C. Lee, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 1744 (1990).

<sup>33</sup> D. L. Taylor, J. Reidler, J. A. Spudich, and L. Stryer, *J. Cell Biol.* **89**, 363 (1981).

<sup>34</sup> E. D. Matayoshi, G. T. Wang, G. A. Krafft, and J. Erickson, *Science* **247**, 954 (1990).

<sup>35</sup> B. W. van der Meer, G. Coker III, and S. Y. Chen, "Resonance Energy Transfer." VCH, New York, 1994.

<sup>36</sup> C. R. Cantor and P. R. Schimmel, "Biophysical Chemistry," Vol. 2, pp. 448–455. Freeman, San Francisco, California, 1980.

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FRET to measure dynamic processes, including enzymatic activity such as HIV proteolysis<sup>34</sup> or molecular assembly such as actin assembly.<sup>1,33</sup> In these cases the distance between two points changes as a function of some dynamic process; the ends of a polypeptide separate after cleavage by HIV, for example. As long as the dynamic process is not too fast (long enough to acquire a reasonable fluorescent signal—roughly, a few minutes with ease or, with effort, subsecond), the process can be monitored by FRET.

Yet another application of FRET is to measure rates of diffusion and distances of closest approach. In these cases, one uses a long-lived donor (lifetime on the order of a microsecond to millisecond). During the excited state lifetime of the donor, the donor and/or acceptor can diffuse. If the distance which they can diffuse during the donor lifetime is on the order of the average distance separating the donor and acceptor, the amount of energy transfer will depend on the diffusion coefficients, and hence measurements of energy transfer can shed light on this quantity. If the average distance is considerably less than the diffusional distance, the donor and acceptor approach each other many times during the donor lifetime, and energy transfer will depend mostly on the distance of closest approach.

A third and relatively new application of FRET is the generation of new compound dyes with spectral characteristics that combine the best of both dyes. The idea is to attach covalently a donor and acceptor together in close proximity to one another. In the simplest case, where the absorption or emission properties of the individual dyes do not change, the absorption characteristic of the compound dye is the sum of the two individual dyes. At the same time, the emission is dominated by the acceptor since almost all of the energy absorbed by the donor is transferred to the acceptor. This results in dyes having potentially large Stokes shifts (the sum of the donor and acceptor Stokes shifts) and excellent quantum yields. So far, this work has mainly been applied to phycobiliproteins and DNA dyes.<sup>39-42</sup>

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l (1990).  
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<sup>37</sup> R. H. Fairclough and C. R. Cantor, this series, Vol. 48, p. 347.

<sup>38</sup> L. Stryer, *Annu. Rev. Biochem.* **47**, 819 (1978).

<sup>39</sup> A. N. Glazer and L. Stryer, *Biophys. J.* **43**, 383 (1983).

<sup>40</sup> S. C. Benson, P. Singh, and A. N. Glazer, in "Human Genome Program, Contractor-Grantee Workshop III," Santa Fe, New Mexico, U.S. Department of Energy, Publ. ORNL/M-2588, 1993.

<sup>41</sup> S. C. Benson, P. Singh, and A. N. Glazer, *Nucleic Acids Res.* **21**, 5727 (1993).

<sup>42</sup> R. P. Haugland, "Molecular Probes, Inc., Catalog" (K. D. Larison, ed.), 5th Ed., Molecular Probes, Eugene, Oregon, 1992-1994.

### Choice of Technique

Alternative techniques which give some of the same information as FRET include X-ray crystallography, nuclear magnetic resonance (NMR), cryoelectron microscopy, and biochemical methods such as gel-shift assays and cross-linking studies. Briefly, X-ray crystallography and NMR both produce potentially complete structural information but require large quantities of material. For *in vitro* studies, FRET is often used to get initial structural information, with the complete solution coming later from X-ray crystallography or NMR. X-Ray crystallography and NMR are limited to *in vitro* measurements and can analyze only relatively small molecules, restrictions which do not apply to FRET. In addition, in the case of X-ray crystallography, one is also faced with the difficult problem of crystallization and isomorphous replacement.

Cryoelectron microscopy achieves the high resolution of electron microscopy while minimizing some of the sample preparation artifacts associated with drying, staining, and interaction with the solid support. The technique has been shown to reproduce structural features of viruses, some protein crystals, and even more flexible samples such as DNA.<sup>43</sup> The technique is limited to using thin (100 nm) samples, contrast is fairly low, and aggregation can occur during the cooling period. A number of reviews on cryoelectron microscopy have appeared.<sup>43-47</sup>

Gel-shift assays, in which the mobility of a sample in a polyacrylamide gel is a function of the structure of the molecule (e.g., bent or straight, compact or extended) can supply some of the same information as FRET. This is because alterations in molecular shape can lead to changes in gel mobility and also changes in the distance (and hence energy transfer) between two site-specifically placed dyes (e.g., in DNA<sup>17,48</sup>). Sample preparation in the gel-shift techniques is generally easier, since no labels need be attached, but the technique has the disadvantage that the complex must be stable over the course of hours and structural changes must be inferred from changes in mobility, which are not well understood theoretically.

Cross-linking studies, like gel-shift assays, have the advantage that site-specific labels need not be introduced into the macromolecule. On the

<sup>43</sup> J. Dubochet, M. Adrian, I. Dustin, P. Furrer, and A. Stasiak, this series, Vol. 211, p. 507.

<sup>44</sup> R. H. Wade and D. Chretien, *J. Struct. Biol.* **110**, 1 (1993).

<sup>45</sup> R. Schroder, W. Hofmann, J. Menetret, K. Holmes, and R. Goody, *Electron Microsc. Rev.* **5**, 171 (1992).

<sup>46</sup> K. Meller, *Electron Microsc. Rev.* **5**, 341 (1992).

<sup>47</sup> P. Flicker, R. Milligan, and D. Applegate, *Adv. Biophys.* **27**, 185 (1991).

<sup>48</sup> U. K. Snyder, J. F. Thompson, and A. Landy, *Nature (London)* **341**, 255 (1989).

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other hand, it is not always clear where the cross-linking is taking place. Furthermore, if cross-linking does not occur, this may be because the sites are not in close proximity or because the sites are not chemically reactive. Finally, if flexible chemical linkers are used, the distance determination is limited to saying that the two points of interest are less than or equal to the maximal extension of the cross-linker. A number of reviews on cross-linking techniques have appeared.<sup>49-52</sup>

### Problems

It is also important to understand the limitations of FRET. Although these are explored in some detail later, the most important drawback of FRET is its limited ability to measure absolute distances. It is quite good at measuring relative distances, namely, whether two points are closer together under condition A than condition B. The problem is that the efficiency of energy transfer depends not only on the distance between the donor and acceptor, but on the relative orientation of the dyes as well, a factor which is often not precisely known. Even when measuring relative distances one must take care to ensure that the orientation factor does not change between the two systems one is comparing. Unfortunately, this orientation factor can be significant, multiplying the fitted-distance anywhere from 0 to  $4^{1/6} = 1.26$ . Polarization measurements on the donor and acceptor can be made which constrain this factor, but rarely do they eliminate all uncertainty. In addition, FRET is limited in its ability to measure absolute distances because there is usually uncertainty in the exact position of the FRET dyes owing to flexibility in the linker arm used to attach the dyes. For these reasons FRET is more easily and reliably used as a measure of relative distance.

A second problem with FRET is the very sharp distance dependence. This has two drawbacks: (1) it is difficult to measure relatively long distances because the signal is very weak, and (2) the signal tends to be "all or none," that is, if the two points are less than a certain characteristic distance (this distance, known as  $R_0$ , is the distance at which 50% of the energy is transferred and is a function of the particular dyes chosen), almost all of the energy is transferred, but if greater than this distance, very little energy is transferred. It is therefore helpful to have some estimate of the distance of interest before a FRET measurement is undertaken.

<sup>49</sup> J. Brunner, *Annu. Rev. Biochem.* **62**, 483 (1993).

<sup>50</sup> J. Brunner, this series, Vol. 172, p. 628.

<sup>51</sup> M. Brinkley, *Bioconjugate Chem.* **3**, 2 (1992).

<sup>52</sup> S. S. Wong and L. J. C. Wong, *Enzyme Microb. Technol.* **14**, 866 (1992).

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### Underlying Principles: Förster's Theory of Dipole-Dipole Interaction

Here we present a brief review of the physical principles underlying fluorescence energy transfer. The theory was developed primarily by Förster and extended by Dexter.<sup>53,54</sup> Förster did some early experimental studies,<sup>55</sup> and Stryer and Haugland convincingly showed that fluorescence energy transfer could be used as a "molecular ruler" to measure distances.<sup>19</sup> Emphasis is on developing an intuitive feel for the important relevant parameters. Both a classical and a quantum mechanical approach are given.

In FRET, a fluorescent donor molecule transfers energy to an acceptor molecule, which is usually but not necessarily a fluorescent molecule. The mechanism is a nonradiative induced dipole-induced dipole interaction<sup>36</sup>: "nonradiative" because no photons are "passed" between the dye molecules; "dipole-dipole" because each dye molecule acts like a classical (or quantum mechanical) dipole antenna, emitting and absorbing energy; "induced" because the dipoles are not permanent but are a result of electric fields which create them. On energy transfer, the signal in a FRET experiment is a decrease in the fluorescence intensity and lifetime of the donor and, if the acceptor is also fluorescent, an increase in the fluorescence of the acceptor. The changes are measured by comparing the fluorescence of a complex containing both donor and acceptor to that of complexes containing only donor or only acceptor. The fluorescence of the donor decreases in the presence of acceptor because some of the energy goes to the acceptor instead of into the radiation (or photon) field. The lifetime of the donor also decreases because the energy transfer to the acceptor is another pathway for the excited state to decay to the ground state.

The efficiency of energy transfer ( $E$ ), which is defined as the fraction of donor molecules de-excited via energy transfer to the acceptor, therefore equals

$$I_{D_A} = 0.5 I \quad E = (1 - I_{D_A}/I_D) = 1 - \tau_{D_A}/\tau_D \quad (1)$$

where  $I_{D_A}$  and  $\tau_{D_A}$  are the intensity and lifetime, respectively, of the donor in the presence of acceptor, and  $I_D$  and  $\tau_D$  in the absence of acceptor. The efficiency of energy transfer can also be measured by looking at the increase in fluorescence of the acceptor:

$$E = \frac{(I_{A_D}/I_A - 1)(\epsilon_A/\epsilon_D)}{\text{exc. population} \times \text{efficiency of absorption}} \quad (2)$$

<sup>53</sup> T. Förster, *Mod. Quantum. Chem. Lect. Istanbul Int. Summer Sch.* (1965).

<sup>54</sup> D. L. Dexter, *J. Chem. Phys.* **21**, 836 (1953).

<sup>55</sup> T. Förster, *Z. Naturforsch. A: Astrophys. Phys. Phys. Chem.* **4**, 321 (1949).

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where  $I_{A_D}$  is the emission of the acceptor in the presence of the donor (consisting of fluorescence arising from energy transfer and from direct excitation of the acceptor) and  $I_A$  is the fluorescence of the acceptor-only labeled sample (consisting of fluorescence arising from direct excitation only);  $\epsilon_A$  and  $\epsilon_D$  are the molar extinction coefficients of the acceptor and donor, respectively, at the wavelength of excitation. Equations (1) and (2) assume complete labeling; in other words, the doubly labeled complex is completely labeled with donor and acceptor. They can be readily modified to account for incomplete labeling.<sup>56</sup>

To get distance information from these experimental parameters, one needs to know how the efficiency of energy transfer depends on distance. Förster showed that

$$E = 1/(1 + R^6/R_0^6) \quad (3)$$

where  $R$  is the distance between the donor and acceptor and  $R_0$  is a characteristic distance, typically 10–50 Å, related to properties of the donor and acceptor.<sup>36,53</sup> From Eq. (3) it is easy to see that  $R_0$  is the distance at which 50% of the energy is transferred:

$$R_0 = (8.79 \times 10^{-5} J q_D n^{-4} \kappa^2)^{1/6} \quad (\text{in } \text{\AA}) \quad (4)$$

$$J = \int \epsilon_A(\lambda) f_D(\lambda) \lambda^4 d\lambda / \int f_D(\lambda) d\lambda \quad (\text{in } M^{-1} \text{ cm}^{-1} \text{ nm}^4) \quad (5)$$

where  $J$  is the normalized spectral overlap of the donor emission ( $f_D$ ) and acceptor absorption ( $\epsilon_A$ ),  $q_D$  is the quantum efficiency (or quantum yield) for donor emission in the absence of acceptor ( $q_D$  is the number of photons emitted divided by number of photons absorbed),  $n$  is the index of refraction (typically 1.3–1.4), and  $\kappa^2$  is a geometric factor related to the relative angle of the two transition dipoles.

Figure 1 shows an example of the spectrum of a commonly used donor and acceptor and the intensity changes which occur with energy transfer. Figure 2 shows the experimental verification of the  $R^{-6}$  dependence of energy transfer using dansyl as a donor and naphthyl as an acceptor, separated by a series of rigid, polyproline linkers.

To generate an intuitive feeling for Eqs. (1)–(5), we first show how the form of Eq. (3) for the fraction of energy transferred is physically reasonable, then we show the sixth power dependence. Finally, we discuss the parameters which determine  $R_0$  in Eqs. (4) and (5).

### Form of Equation

We first show that the form of the energy transfer efficiency qualitatively looks like  $1/[1 + f(r)]$  where  $f(r)$  is some function of the distance,

<sup>56</sup> B. Epe, K. G. Steinhauser, and P. Woolley, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 2579 (1983).



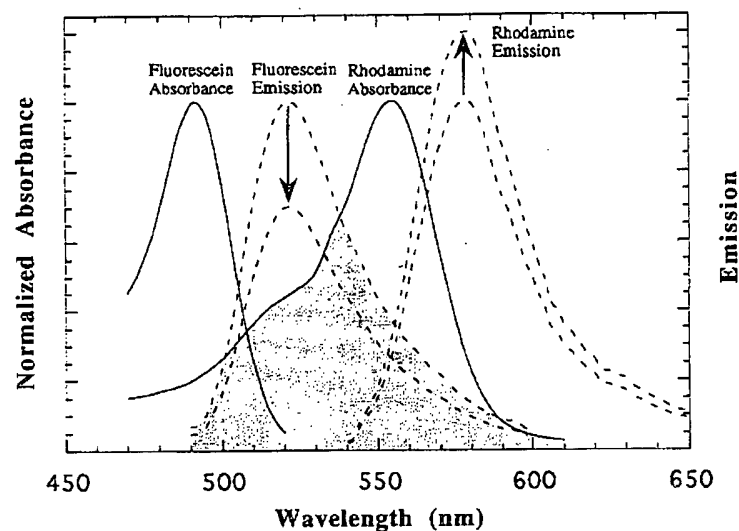


FIG. 1. Spectral characteristics and changes of the donor fluorescein and acceptor tetramethylrhodamine undergoing energy transfer. The donor intensity decreases and the acceptor is sensitized with energy transfer. The spectral overlap which makes energy transfer possible is shown in gray. The absorbance and emission intensities are normalized for display purposes. The  $R_0$  for the pair is approximately 45 Å.

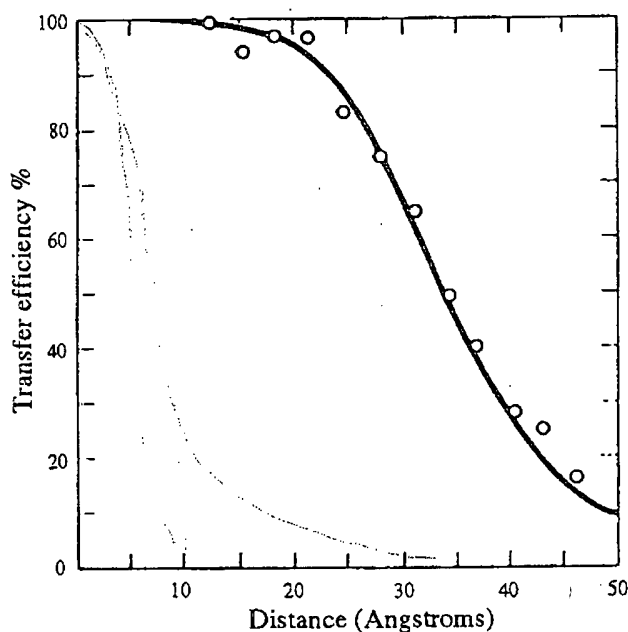


FIG. 2. Experimental verification of Förster's theory of fluorescence energy transfer. Energy transfer was studied with a series of end-labeled oligopeptides, dansyl-(polyproline)<sub>n</sub>-naphthyl. The solid line is a fit to the data with Eq. (3) showing the  $R^{-6}$  dependence. (From Stryer and Haugland.<sup>19</sup>)

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$r$ , between the donor and acceptor. Consider a donor which has been excited by light. The excited state will decay with rate  $k_{nd} + k_{et}$ , where  $k_{nd}$  is the sum of all distance-independent rates such as fluorescence ( $k_f$ ) and heat ( $k_h$ ) ( $k_{nd} = k_f + k_h = \tau_D^{-1}$ ) and  $k_{et}$  is the distant-dependent rate of energy transfer to an acceptor.

The fraction of donor molecules giving energy to the acceptor is therefore just

$$E = k_{et}/(k_{et} + k_{nd}) = 1/(1 + k_{nd}/k_{et}) = 1/(1 + 1/\tau_D k_{et}) \quad (6)$$

which is the form of Förster's equation [Eq. (3)] if  $k_{et}$  depends on  $R^{-6}$  and  $R_0$  is related to constants in  $k_{et}$  and  $k_{nd}$ . In addition, the excited state donor lifetime decreases from  $1/k_{nd}$  ( $\tau_D$ ) without the acceptor, to  $1/(k_{nd} + k_{et})$  ( $\tau_{DA}$ ) with the acceptor, directly leading to Eq. (1). When the acceptor is nearby, the energy transfer rate is fast compared to other decay pathways and most of the energy is transferred. If the acceptor is farther away this rate is less and the efficiency of energy transfer decreases. For later reference, we note that combining Eq. (3) and Eq. (6) yields

$$k_{et} = \tau_D^{-1}(R/R_0)^6 \quad (7)$$

Why is the distance dependence of energy transfer  $R^{-6}$ ? It comes about because the extent of energy transfer depends on the square of the electric field produced by the donor, and this field decays as  $R^{-3}$  at distances relevant in FRET. First we examine the donor electric field. The electric field of the donor arises because the incident excitation light induces electrons in the donor to oscillate (or, in quantum mechanics terms, induces transitions). This creates an induced, electric dipole moment in the donor, which creates its own, characteristic electric field. The dipole field of the donor has two parts, one which dies away like  $1/R$ , the other which decays like  $1/R^3$ . Far away from the molecule, the  $1/R$  term dominates. This is called the radiation field and is what we see as fluorescent photons. (The energy carried away goes like the square of the electric field, and therefore drops off as  $1/R^2$ , as it must to conserve energy.) Close up, however, within a wavelength of light, the  $1/R^3$  term dominates. This field is the one of interest in FRET. It does not carry away energy, that is, does not radiate, and so sometimes FRET is called nonradiative energy transfer. (In very rare instances, when using lanthanides as donors, for example, the donor may act partially like a magnetic dipole<sup>57</sup> or, in the solid phase, even like an electric quadrupole<sup>58-60</sup> instead of, or in addition

<sup>57</sup> J.-C. G. Bunzli and G. R. Choppin (eds.), "Lanthanide Probes in Life, Chemical and Earth Sciences: Theory and Practice," Elsevier, New York, 1989.

<sup>58</sup> R. Reisfeld and L. Boehm, *J. Solid State Chem.* **4**, 417 (1972).

<sup>59</sup> E. Nakazawa and S. Shionoya, *J. Chem. Phys.* **47**, 3211 (1967).

<sup>60</sup> R. Reisfeld, E. Greenberg, and R. Velapoldi, *J. Chem. Phys.* **56**, 1698 (1972).

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To understand why energy transfer is proportional to the square of the  $R^{-3}$  field of the donor, we must understand how the acceptor interacts with (takes energy from) the donor electric field. If an acceptor molecule is in this close-up electric field, its electrons will be induced to oscillate, creating an induced dipole moment,  $p_A$ , in the acceptor (just as an induced dipole was formed in the donor by the incident electric field of the exciting light). The size of the dipole is related to the size of the electric field,  $E_D$ , creating it:  $p_A = \alpha_A E_D$ , where  $\alpha_A$  is the polarizability of the acceptor and is a measure of how easily the electrons can be made to oscillate.

What is the fraction (or efficiency) of energy transferred? It is simply the energy absorbed by the acceptor from the donor, divided by the energy absorbed by the donor from the excitation light. The latter term is independent of any distances of interest and so is irrelevant here. Moreover, the amount of energy absorbed by the acceptor is just  $p_A \cdot E_D = \alpha_A E_D^2$ . Consequently, because  $E_D$  decays as  $R^{-3}$ , the amount of energy absorbed by the acceptor is a function of  $R^{-6}$  between donor and acceptor.

Of course the proper treatment of energy transfer is via quantum mechanics. The analysis is very straightforward, and an excellent outline is presented by Cantor and Schimmel.<sup>36</sup> The excitation light induces transitions in the donor to an excited (singlet) state. This decays rapidly to the lowest excited state. The donor can then relax either via fluorescence, nonradiative processes, or interaction with the acceptor via a dipole-dipole interaction (see Fig. 3). The Hamiltonian or energy of interaction between the donor and acceptor is

$$H = (\mu_D \cdot \mu_A)/R^3 + (\mu_D \cdot \mathbf{R})(\mu_A \cdot \mathbf{R})/R^5 \quad (8)$$

where  $\mu_D(\mu_A)$  is the transition dipole moment of the donor (acceptor) and  $\mathbf{R}$  is the vector separating their centers. According to Fermi's rule, the rate of inducing transitions is proportional to the square of the Hamiltonian matrix element between final and initial states:

$$k_{et} \propto [ \langle D^* | \langle A | [ (\mu_D \cdot \mu_A)/R^3 - 3(\mu_D \cdot \mathbf{R})(\mu_A \cdot \mathbf{R})/R^5 ] | D \rangle | A^* \rangle ]^2 \quad (9)$$

where the initial state is the product of the excited state of the donor ( $\langle D^* |$ ) and the ground state of the acceptor ( $\langle A |$ ) and the final state is the product of the donor ground state ( $| D \rangle$ ) and acceptor excited state ( $| A^* \rangle$ ). We can write the wave function as this simple product because we assume the coupling between donor and acceptor is weak, and so the individual wave functions are not much perturbed. (If this is not the case, one gets into exciton coupling where the absorption spectra of the individual dyes change in the donor-acceptor complex). Only those donor and acceptor

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wave functions with nearly the same energy will significantly contribute to the rate; this is the resonance condition of FRET. The dot products can be explicitly written and Eq. (9) separated into those quantities depending on the donor wave functions and those on the acceptor wave functions, and those depending on the relative orientation:

$$k_{et} \propto R^{-6} \langle D^* | \mu_D | D \rangle^2 \langle A | \mu_A | A^* \rangle^2 [\langle D^* | A | (\cos \theta_{DA} - 3 \cos \theta_D \cos \theta_A) | D \rangle | A^* \rangle]^2 \quad (10)$$

The rate is therefore proportional to the square of the transition dipole moments of the acceptor and donor, which can be related to the absorption and emission properties of each dye, respectively.<sup>36</sup> The rate also depends on a geometric factor.

Combining Eq. (10) and Eq. (7) yields Förster's equation [Eq. (3)] where  $R_0$  is a function of the acceptor absorption cross section, the donor emission efficiency, and also the relative angles of the donor and acceptor dipoles [see Eqs. (4) and (5)].

#### Parameters in $R_0$

We can now understand the parameters which enter into  $R_0$  [Eq. (4)].  $R_0$  is a measure of how well the donor and acceptor can transfer energy to one another, where a large  $R_0$  indicates that the donor and acceptor

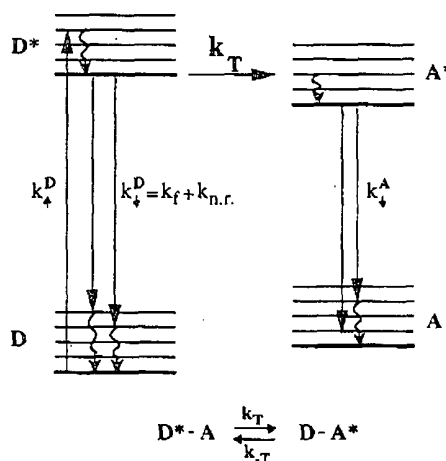


FIG. 3. Jablonski energy level diagram. The donor is excited and rapidly drops to the lowest vibrational level of the excited state, where it can radiatively (primarily via fluorescence) or nonradiatively decay to the ground state, or transfer energy to the acceptor. Only those levels of the donor and acceptor with similar energies contribute significantly to the transfer rate. Once the acceptor is excited, rapid vibrational relaxation prevents back transfer. The acceptor then decays to the ground state via fluorescence or heat.

can transfer energy efficiently even if they are relatively far apart. To transfer energy from donor to acceptor, the electric field produced by the donor must be at a frequency (or wavelength) that can induce transitions in the acceptor (or, classically, a frequency which can efficiently drive the electrons in the acceptor). Consequently,  $R_0$  depends on the spectral overlap term,  $J$ , which is a measure of how well the donor fluorescence frequency (or wavelength) and acceptor absorbance overlap in wavelength. Why is there a  $\lambda$  dependence in the  $J$  term? The answer is that the electric field dies off as  $(\lambda/r)^3$ . If  $\lambda$  is large (further to the red) the electric field drops off more slowly, and energy transfer can occur at farther distances. Mathematically, the  $\lambda^4$  power comes about because  $\mu_A^2$  is proportional to  $\epsilon_A \lambda$  and  $\mu_D^2$  is proportional to  $\lambda^3 q_D / \tau_D$ .<sup>36</sup> Note that the dependence of  $\mu_D^2$  on  $\tau_D^{-1}$  eliminates the donor lifetime dependence from  $R_0$ . This is expected because, although the rate depends on the lifetime, a longer lifetime means a slower rate integrated over a longer time. The index of refraction enters into  $R_0$  because the size of the electric field produced by the donor is modified by the polarizability of the medium, which is directly related to the index of refraction. Finally, the quantum yield of the donor,  $q_D$ , enters because it is a measure of how well the donor converts the energy it has absorbed into an electric field (as opposed to converting the energy to phonons, or heat). (A donor with a high quantum yield efficiently creates a large electric near-field, which is relevant for FRET, as well as a large electric far-field, the latter being fluorescent photons.)

The last and most troubling term in  $R_0$  is  $\kappa^2$ , which arises because the efficiency of energy transfer depends on the relative orientation of the two dyes (the  $\mu_D \cdot \mu_A$  term) and the relative orientation in space [the  $3(\mu_D \cdot \mathbf{R})(\mu_A \cdot \mathbf{R})$  term]. The expression for  $\kappa^2$  is

$$\kappa^2 = (\cos \theta_{DA} - 3 \cos \theta_D \cos \theta_A)^2 \quad (11)$$

where  $\theta_{DA}$  is the angle between the donor and acceptor transition dipole moments and  $\theta_D$  ( $\theta_A$ ) is the angle between the donor (acceptor) transition dipole moment and the  $\mathbf{R}$  vector joining the two dyes.

One can immediately see that  $\kappa^2$  can vary from 0 if all angles are perpendicular to 4 if all angles are parallel. If the orientation of the dipoles is random, because they are moving rapidly (within the donor lifetime) then  $\kappa^2 = 2/3$ . This assumption is often made, even if not strictly true, and accounts for much of the uncertainty in FRET measurements. If just the donor or just the acceptor is randomized, then  $1/3 < \kappa^2 < 4/3$ . In this case the uncertainty in measured distance is approximately  $\pm 11\%$ .<sup>38</sup> In reality, what usually happens is that the dyes undergo fast, restricted motion such that  $\kappa^2$  approaches 2/3, with some uncertainty remaining.

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Dale and Eisinger have analyzed the effect of rotational mobility<sup>61</sup>; Stryer presents an analysis of the errors introduced by assuming  $\kappa^2 = 2/3$ <sup>38</sup>; and van der Meer *et al.* present a treatment on the effects of restricted rotational and translational diffusion.<sup>62</sup> Experimentally, one determines the rotational mobility of the dyes by a steady-state or time-resolved fluorescence depolarization experiment.<sup>63</sup>

In practice,  $R_0$  is often measured from a model system (see, e.g., Fig. 2) and assumed to apply to the system of interest, or  $R_0$  is calculated from Eqs. (4) and (5), assuming a value  $\kappa^2 = 2/3$ .

### Labeling

Perhaps the most difficult aspect of FRET is the problem of labeling the sites of interest with the appropriate dyes. One must choose dyes that are spectrally compatible and that can be site specifically labeled without significantly perturbing the original structure of the molecule of interest. In general it is also important that the sample be completely labeled with both donor and acceptor, or at least that the extent of labeling be known. Analyses have appeared which take into account incomplete labeling.<sup>2,64</sup> Waggoner ([15] in this volume) reviews fluorescent labeling, and Brinkley has reviewed techniques for labeling proteins with dyes, haptens, and cross-linking reagents.<sup>51</sup>

### Naturally Occurring Fluorophores

The ideal situation is if the biological molecule is fluorescent, or can be made so with slight modification. In proteins, tryptophan is a naturally occurring amino acid which makes a reasonable donor. Tyrosine is also fluorescent but is rarely a good donor to an external acceptor because it is often quenched by tryptophans in the protein.<sup>36</sup> Beardsley and Cantor have used the fluorescent Y-base associated with yeast phenylalanine transfer RNA.<sup>25</sup> For DNA and RNA there are a number of fluorescent nucleotide analogs.<sup>38,42</sup> Yet another possibility is if the protein binds a ligand which is either fluorescent or can be made so. In metal-binding proteins, zinc can be replaced with cobalt, which can act as a good acceptor because of wide visible absorbance.<sup>22,65</sup> Terbium, another metal, can act

<sup>61</sup> R. E. Dale, J. Eisinger, and W. E. Blumberg, *Biophys. J.* **26**, 161 (1979).

<sup>62</sup> B. W. van der Meer, M. A. Raymer, S. L. Wagoner, R. L. Hackney, J. M. Beechem, and E. Gratton, *Biophys. J.* **64**, 1243 (1993).

<sup>63</sup> J. R. Lakowicz, "Principles of Fluorescence Spectroscopy." Plenum, New York, 1983.

<sup>64</sup> J. R. Lakowicz, I. Gryczynski, W. Wiczk, J. Kusba, and M. L. Johnson, *Anal. Biochem.* **195**, 243 (1991).

<sup>65</sup> S. A. Latt, D. S. Auld, and B. L. Vallee, *Proc. Natl. Acad. Sci. U.S.A.* **67**, 1383 (1970).

as an excellent donor especially if there is a tryptophan nearby. (The tryptophan, in effect, increases the absorption cross section of the terbium by absorbing light and passing energy to the terbium, which by itself, has very weak absorbance.)<sup>66</sup> Terbium and europium have also been used as isomorphous replacements for calcium<sup>22</sup> and in metal-binding engineered proteins.<sup>67,68</sup>

### Fluorescent Dyes

There is a large number of dyes to choose from, many of which are listed in the Molecular Probes (Eugene, OR) catalog.<sup>42</sup> Ideally, one picks a pair with  $R_0$  equal to the distance to be measured, since small changes in distance around  $R_0$  lead to large changes in signal. However, because the distance is generally unknown, or only approximately known, it is wise to pick a donor-acceptor pair with a large  $R_0$ , equal to or larger than the distance to be measured. If necessary,  $R_0$  can be decreased by adding reagents such as iodine that reduce the donor quantum yield or by choosing an acceptor with poorer spectral overlap to the donor.

To achieve a large  $R_0$  one wants a donor with a high quantum yield of long-wavelength emission and an acceptor with a large absorbance at the donor fluorescence wavelengths. Unfortunately, as the emission becomes more red, the quantum yield tends to drop, although there are some promising new dyes with reasonable quantum yields in the red, such as La Jolla Blue,<sup>69</sup> CY-5,<sup>70,71</sup> and Bodipy<sup>42</sup> as well as nucleic acid stains such as BOBO and POPO,<sup>42</sup> and also the phycobiliproteins.<sup>42</sup>

Increasing  $R_0$  and thereby getting a large signal is only part of the strategy. Minimizing background is also important. When measuring donor quenching or sensitized emission, it is desirable to have little spectral overlap between donor and acceptor fluorescence. For sensitized emission, it is also desirable for the acceptor not to be excited directly by the excitation light. This requires the acceptor absorbance to be small where the donor absorbance is large. Finally, to maximize the sensitized emission signal, it is desirable for the acceptor to have a good quantum yield.

The ideal situation is therefore when both donor and acceptor have high quantum yields and large Stokes shifts. Unfortunately a large Stokes

<sup>66</sup> P. Cioni, G. B. Strambini, and P. Degan, *J. Photochem. Photobiol. B* **13**, 289 (1992).

<sup>67</sup> J. P. MacManus, C. W. Hogue, B. J. Marsden, M. Sikorska, and A. G. Szabo, *J. Biol. Chem.* **265**, 10358 (1990).

<sup>68</sup> I. D. Clark, J. P. MacManus, D. Banville, and A. G. Szabo, *Anal. Biochem.* **210**, 1 (1993).

<sup>69</sup> R. Devlin, R. M. Studholme, W. B. Dandliker, E. Fahy, K. Blumeyer, and S. S. Ghosh, *Clin. Chem.* **39**, 1939 (1993).

<sup>70</sup> H. Yu, L. Ernst, M. Wagner, and A. Waggoner, *Nucleic Acids Res.* **20**, 83 (1992).

<sup>71</sup> R. B. Mujumdar, L. A. Ernst, S. R. Mujumdar, C. J. Lewis, and A. S. Waggoner, *Bioconjugate Chem.* **4**, 105 (1993).

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shift generally implies a small quantum yield, so there is a trade-off in these properties. (In the section on future directions, we discuss an energy transfer scheme using lanthanides as donors and organic chromophores as acceptors which gets around these problems.)

Perhaps the most popular donor is fluorescein, which often has a quantum yield exceeding 0.5. It can be used with eosin ( $R_0 = 50\text{--}54 \text{ \AA}^{14}$ ), chlorofluorescein ( $\approx 50 \text{ \AA}$ ), tetramethylrhodamine ( $R_0 = 45 \text{ \AA}^{16}$ ), tetraethylrhodamine ( $R_0 = 40 \text{ \AA}^{37}$ ), or Texas Red ( $R_0$  not reported but  $\ll 40 \text{ \AA}$ ). (All  $R_0$  values are approximate because they depend on the donor quantum yield and emission spectra which can change depending on solvent conditions.) In this series, as  $R_0$  decreases, the spectral separation increases. Greater spectral separation is especially helpful if one is measuring the sensitized emission of the acceptor (see measurement section below) or if only a relatively small percentage of molecules have both donor and acceptor bound or are capable of transferring energy. (See the FRET work of McConnell and co-workers on conformational changes in high mobility group (HMG) proteins for an example of such a case.<sup>23</sup>

Tetramethylrhodamine is perhaps the most popular acceptor with fluorescein because of its large  $R_0$  and because the acceptor fluorescence is somewhat separated from donor fluorescence. Fluorescein fluorescence can be monitored from 500 to 525 nm with no contamination from acceptor fluorescence. A new carbocyanine dye, CY-3, is spectrally very similar to tetramethylrhodamine but is reported to have somewhat higher maximum absorbance.<sup>71-73</sup> Eosin as acceptor has a somewhat higher  $R_0$  than tetramethylrhodamine, but its emission strongly overlaps that of fluorescein, such that there is not even a separable maximum. Fluorescein as donor does have the disadvantage that it may be quenched approximately 50% in proteins,<sup>42</sup> and it has a short, multiexponential (main component of 3 nsec) lifetime which makes donor lifetime measurements difficult, of limited accuracy, and especially problematic if  $R$  is significantly greater than  $R_0$ . The quantum yield of fluorescein is also a strong function of pH below pH 8 and decreases with increasing  $\text{Na}^+$ ,  $\text{Cl}^-$ , and  $\text{Mg}^{2+}$ .<sup>18</sup>

Other dyes such as dansyl and AEDANS are popular donors in protein studies, in part because of the relatively long lifetimes (13–20 nsec), large (150 nm) Stokes shifts, and reasonable quantum yields (0.1–0.5).<sup>42</sup> If steric hindrance is not a problem and a large-sized donor or acceptor can be used, the multichromophoric phycobiliproteins (molecular weight of 104,000 for B- or R-phycoerythrin; 240,000 for allophycocyanin) make excellent donors or acceptors, having extinction coefficients which can exceed

<sup>72</sup> "Biological Detection Catalog." Biological Detections Inc., Pittsburgh, Pennsylvania, 1993.

<sup>73</sup> "Research Organics Inc., Catalog." Cleveland, Ohio, 1993.



$2 \times 10^6$  and quantum yields of 0.68–0.98. They are also available with reactive groups.<sup>42</sup> Fairclough and Cantor present a useful list of donor–acceptor pairs, including spectra and  $R_0$  values,<sup>37</sup> and van der Meer and co-workers<sup>35</sup> are compiling an extensive list.

### Site-Specific Attachment

Fortunately there is a wide variety of dyes that are available with reactive groups. For attachment to amines, the most common reactive groups are succinimide esters and isothiocyanates. The former have generally better coupling efficiency if the coupling can be done in an organic phase. For attachment to synthetic DNA, an amine-modified base can be introduced via commercially available phosphoramidite, either internally or on the 3' or 5' ends.<sup>74–76</sup> Direct attachment to the DNA backbone has also been achieved.<sup>15</sup> Relatively short polypeptides can often be readily labeled, especially if they contain a unique cysteine or amine-containing amino acid.<sup>19,34,77</sup> For proteins, lysines (and to a significantly lesser extent arginines) are available for labeling.<sup>78</sup>

With proteins the problem is generally that there are too many reactive sites. Site-directed mutagenesis can sometimes be used to introduce a free sulfhydryl group which can then be coupled to a dye via an iodoacetamide or a maleimide. An extensive list of proteins which have been labeled with iodoacetamides has been tabulated.<sup>42</sup> Site-directed mutagenesis can also introduce a tryptophan (as donor) if one is not already present. ANS or AEDANS are good acceptors for tryptophan, both yielding an  $R_0$  of 22 Å. Proteins with N-terminal serine or threonine can also be specifically labeled.<sup>37,38</sup> Objects which can be biotinylated can be labeled with fluorescent avidin or streptavidin. More generally, fluorescently labeled antibodies can be used to bind to a wide variety of biological substrates.

The reactive groups are often attached to the dyes via a *n*-carbon linker, where *n* typically ranges from 2 to 12. The linker often allows relatively free rotation of the dye, which minimizes uncertainty in  $\kappa^2$ . It can also minimize quenching of the dye, especially if the dye is quenched by a hydrophobic environment. The linker, however, has the disadvantage of adding uncertainty to the exact position of the dye. In general, the

<sup>74</sup> "Clonetech Catalog." Palo Alto, California, 1993.

<sup>75</sup> "Glen Research Catalog." Sterling, Virginia, 1993.

<sup>76</sup> "Peninsula Laboratories Catalog." Belmont, California, 1993.

<sup>77</sup> Y. Pouny, D. Rapaport, A. Mor, P. Nicolas, and Y. Shai, *Biochemistry* 31, 12416 (1992).

<sup>78</sup> T. E. Creighton, "Proteins: Structures and Molecular Properties," 2nd Ed., Freeman, New York, 1993.

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minimal length that allows free rotation of the dye and does not cause quenching is desirable. A six-carbon linker is a good starting point.

### *Testing for Altered Structures*

It is important to check whether the introduction of fluorescent labels alters the macromolecular structure. For DNA, testing the hybridization melting temperature is a crude measure. For proteins, comparing the function (enzymatic activity, binding constant, etc.) of the labeled versus unlabeled molecule is an excellent check.

### *How to Measure Energy Transfer*

There are several ways to measure the amount of energy transfer: (1) decrease in donor intensity or quantum yield; (2) increase in intensity of acceptor emission (sensitized emission); (3) decrease in lifetime of donor; (4) decrease in photobleaching of donor; and (5) change in lifetime of sensitized emission (discussed in the section on future directions below).

### *Donor Intensity*

The simplest way to measure energy transfer is to measure the decrease in fluorescence of the donor in the presence and absence of acceptor. The fractional decrease in the donor fluorescence with the acceptor present is equal to the efficiency of energy transfer [see Eq. (1)]. The only instrumentation necessary is a steady-state fluorimeter. Besides simplicity, the steady-state measurement has the advantage that even a relatively small amount of energy transfer can be measured. If care is taken in measuring concentrations and in the spectroscopy, a 5% decrease in fluorescence is measurable: this corresponds to a distance of  $1.6 R_0$ . One caution is that the optical density (OD) of the sample must be kept sufficiently low that no appreciable absorption of the donor fluorescence takes place; less than 0.02 OD is recommended. Another caution is that the donor and acceptors should be chosen so that there is a region of donor-only fluorescence. Although it is not necessary to have an acceptor which is fluorescent in this experiment, it is important confirmation that energy transfer is taking place because an increase in acceptor emission can arise only from energy transfer (assuming the optical density is low enough), whereas donor quenching can arise from several trivial sources. With a fluorescent acceptor, one can also measure the polarization of the acceptor emission, which tells about its rigidity and hence limits the uncertainty in  $\kappa^2$ .

In addition to measuring the decrease in donor fluorescence, if one wants to calculate  $R_0$ , it is necessary to measure the quantum yield of

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the donor in the absence of acceptor, and also to measure the steady-state or time-resolved polarization of both donor and acceptor. Quantum yield measurements are generally done by comparing total fluorescence of the sample to a reference with known quantum yield.<sup>36,79,80</sup> Polarization measurements are important to limit uncertainty in  $\kappa^2$ , and they are covered by H. van Amerongen and W. S. Struve (this volume [11]) as well as in a book by Lakowicz on fluorescence.<sup>63</sup>

### Sensitized Emission

With the same steady-state fluorimeter, one can also measure the sensitized emission of the acceptor. The efficiency of energy transferred calculated via donor quenching should agree with that calculated by sensitized emission. Sensitized emission can also be particularly useful when measuring long distances or when the sample is inhomogeneous and only a small fraction of the sample contributes to energy transfer.<sup>23</sup> In either case it is helpful if the fluorescence of the acceptor is well separated from that of the donor. In principle, one can measure the energy transfer via sensitized emission based on Eq. (2). In reality, there are experimental difficulties, discussed by Epe *et al.*<sup>56</sup> In particular, for distances much beyond  $R_0$ , the ratio  $I_{AD}/I_A$  approaches unity, and even small errors in measurements lead to large errors in the calculated energy transfer.

A number of workers have attempted to make sensitized emission (and donor quenching) measurements more robust. In general these techniques attempt to reduce the number of independent samples (donor-acceptor, donor only, acceptor only) which must be compared. Fairclough and Cantor cover standard methods.<sup>37</sup> Epe and co-workers<sup>56</sup> developed a technique where a donor-acceptor labeled sample is measured and then enzymatically digested, thereby separating the donor from the acceptor and eliminating energy transfer. Donor quenching or sensitized emission can therefore be made on one sample. Clegg and co-workers have developed an analysis of acceptor emission which yields reproducible results, even when measuring samples with small energy transfer and under significantly different conditions.<sup>2,17,18</sup> They applied the analysis to DNA samples labeled with fluorescein and tetramethylrhodamine, but it should be generally applicable to other FRET pairs. Clegg has outlined the many advantages of the technique.<sup>2</sup> We present a brief outline of the technique.

<sup>79</sup> G. Weber and F. W. J. Teale, *Trans. Faraday Soc.* **53**, 646 (1957).

<sup>80</sup> J. B. Birks, "Photophysics of Aromatic Molecules." Wiley (Interscience), New York, 1970.

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Steady-state emission spectra of a donor-acceptor labeled sample and a donor-only labeled sample are taken. The donor emission is removed from the donor-acceptor emission spectrum by subtracting the normalized donor-only emission spectrum. This leaves the fluorescence of the acceptor due to direct excitation and due to energy transfer (see Fig. 4). Clegg and co-workers call this the "extracted acceptor emission" spectrum,  $F_{em}^A$ . Note that this process does not require the concentration of donor-only sample to be the same as the donor-acceptor sample—only the shape of the donor spectrum is used. This spectrum is divided by a fluorescence value (often the maximum) of an emission spectrum taken on the donor-acceptor complex excited at a wavelength where only the acceptor absorbs (565 nm for fluorescein-tetramethylrhodamine). Alternatively, one can divide by the maximum of the excitation spectrum of the donor-acceptor complex (excitation at 400–590 nm, emission in the range 580–600 nm, for fluorescein-rhodamine). In either case, the resultant ratio spectrum, "(ratio)<sub>A</sub>," is normalized for quantum yield of acceptor, for concentration of total molecules, and for incomplete acceptor labeling.

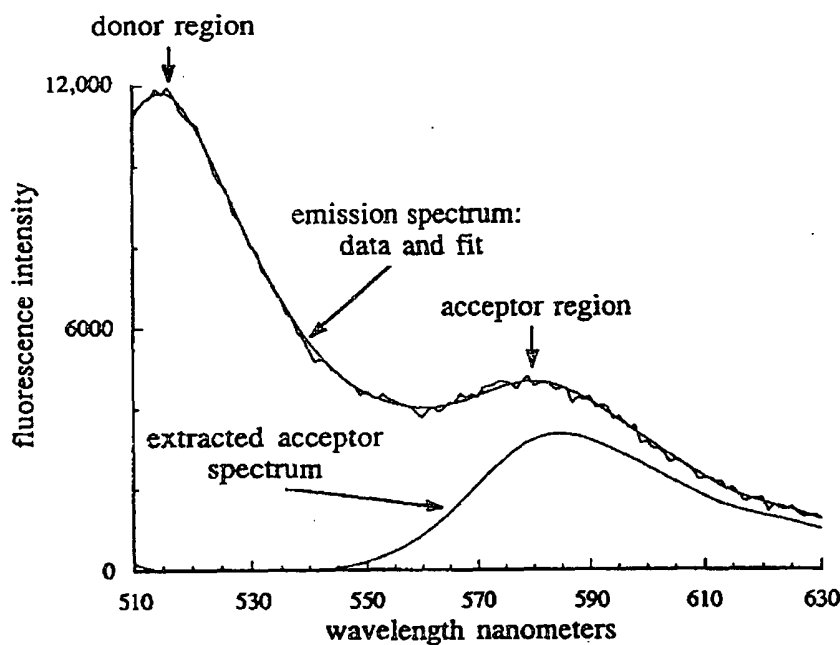


FIG. 4. Emission spectrum of fluorescein-tetramethylrhodamine labeled DNA oligomer. The region where only the donor emits (500–530 nm) is fit to a fluorescein-only spectrum and then subtracted from the entire spectrum, leaving the "extracted acceptor spectrum" consisting of acceptor fluorescence arising from energy transfer and from direct fluorescence. This spectrum is then fit using Eq. (4). The Raman background is subtracted from each fluorescence spectrum. (From R. M. Clegg, this series, Vol. 221, p. 372.)

Clegg and co-workers<sup>2,18</sup> have shown this ratio to be

$$\begin{aligned} (\text{ratio})_A &= F_{\text{em}}^A(\nu_1, \nu') / F_{\text{em}}^A(\nu_2, \nu'') \\ &= \{E \cdot \varepsilon^D(\nu') / \varepsilon^A(\nu'') + \varepsilon^A(\nu') / \varepsilon^A(\nu'')\} [\phi(\nu_1) / \phi(\nu_2)] \end{aligned} \quad (12)$$

where superscripts D and A refer to donor and acceptor,  $\varepsilon$  is the molar extinction coefficient,  $E$  is the efficiency of energy transferred,  $\nu'$  and  $\nu_1$  are the excitation and emission wavelengths, respectively, for the FRET measurement,  $\nu''$  is the excitation wavelength where the acceptor alone absorbs (560 nm for rhodamine), and  $\nu_2$  is the wavelength(s) where the acceptor emission is measured.  $\phi(\nu_i)$  is an "emission spectrum shape function" of the acceptor, where the integral over  $\nu$  is proportional to the quantum yield. It is simply the fluorescence of the acceptor-only sample at wavelength  $\nu_i$ . [Because  $\phi(\nu_i)$  enters only as a ratio, the concentration of acceptor is unimportant.] For the fluorescein-tetramethylrhodamine pair,  $\nu_1$  and  $\nu_2$  are both 585 nm. The second term within the braces,  $\varepsilon^A(\nu') / \varepsilon^A(\nu'')$  can be measured on the acceptor-only sample via absorbance or via an excitation spectrum.

In the scheme developed by Clegg and co-workers,<sup>2,17,18</sup> it is not necessary to compare the intensities of a donor-only or an acceptor-only sample with the doubly-labeled sample; hence, errors in concentration between samples do not lead to errors in measured energy transfer. When comparing samples under different solvent conditions, it is necessary to measure the quantum yield of donor in each case since this parameter enters into  $R_0$ . More general equations can be found which include the effect of incomplete labeling, and for the case when the donor and acceptor absorbances and emissions overlap significantly.<sup>2</sup>

### Donor Lifetime

Measurement of the donor lifetime, which typically is 2–25 nsec, requires adequate time resolution. Two techniques, time-correlated single-photon counting and frequency-domain fluorimetry modulation, can be used (see A. R. Holzwarth, this volume [14]). Excellent books have been written which include discussion of each technique,<sup>63,81</sup> and Lakowicz and co-workers have discussed advances in frequency-domain instrumentation and applications to FRET.<sup>82</sup> Donor lifetime measurements, unlike steady-state measurements, are capable of detecting multiple donor-acceptor transfer efficiencies in the sample. These lead to multiexponential decays. Donor lifetime measurements are also not affected by an inner-filter effect

<sup>81</sup> D. V. O'Connor and D. Phillips, "Time-Correlated Single Photon Counting." Academic Press, London, 1984.

<sup>82</sup> K. W. Berndt, J. R. Lakowicz, and I. Gryczynski, *Anal. Biochem.* **192**, 131 (1991).

where the significant difference in donor-acceptor energy transfer

### Donor Photophysics

A four-photon bleaching technique is particularly sensitive to changes in donor-acceptor energy transfer. It is an alternative to the standard method of measuring energy transfer efficiency by comparing the fluorescence of a donor-only sample with that of a donor-acceptor sample. The constant can be estimated by comparing the fluorescence of a donor-only sample with that of a donor-acceptor sample.

A second method for measuring energy transfer efficiency is by comparing the quantum yield of a donor-only sample with that of a donor-acceptor sample. The quantum yield of a donor-only sample can be measured by integrating the fluorescence of a donor-only sample over the entire emission spectrum. The quantum yield of a donor-acceptor sample can be measured by comparing the fluorescence of a donor-only sample with that of a donor-acceptor sample.

<sup>83</sup> G. Szatmari, *J. Phys. Chem.* **66**, 661 (1962).

<sup>84</sup> U. Kubacki, *Biophys. J.* **61**, 1111 (1991).

where the donor fluorescence is absorbed by the acceptor. Even more significantly, donor lifetime is not sensitive to concentration; therefore, differences in concentration between the donor-only sample and the donor-acceptor sample do not lead to errors in the measurement of energy transfer.

### Donor Photobleaching

A fourth way to measure energy transfer is based on changes in photobleachability of the donor. The technique, pioneered by the Jovin group, is particularly well suited to FRET in a microscope where the high light intensities necessary are readily accessible and where other FRET techniques have yielded only qualitative results.<sup>10-12</sup> (The discussion here is adopted from Ref. 10.) The idea is that the donor photobleaches more slowly if energy transfer to an acceptor is occurring since energy transfer is an alternative pathway for the excited state to give up energy. It can be shown that the fractional change in the photobleaching time constant caused by energy transfer is the same as the fractional change in the fluorescence lifetime of the donor. Thus, in the simplest case, the efficiency of energy transfer is just  $1 - \tau_{bl}/\tau_{bl}'$  where  $\tau_{bl}$  ( $\tau_{bl}'$ ) is the bleaching time constant in the absence (presence) of acceptor. The rate of photobleaching can be easily measured in solution or in a microscope.

A second, related way of measuring energy transfer is based on changes in quantum yield. First a low-light level image is taken of a sample labeled only with donor. The fluorescent intensity at any spot is proportional to the quantum yield and total number of fluorophores. To normalize by the total number of fluorophores (in a manner independent of quantum yield), a high-light level image is taken, and all the fluorescent photons are counted (integrated) until complete photodestruction has occurred. The ratio of the low-light image to the integrated, high-light image is proportional to the quantum yield. The procedure is repeated with a donor-acceptor label sample, and the energy transfer is just the usual 1 minus the ratio of quantum yields [analogous to Eq. (1)]. If a linear camera [e.g., one based on a charge-coupled device (CCD) sensor] is used as the detector, a pixel-by-pixel energy transfer image can be obtained. A number of workers have applied photobleaching-FRET to epitope mapping in T cell lines,<sup>83</sup> visualizing receptor aggregation on the surface of living mast cells,<sup>84</sup> and

<sup>83</sup> G. Szaba, Jr., P. S. Pine, J. L. Weaver, M. Kasari, and A. Aszalos, *Biophys. J.* **61**, 661 (1992).

<sup>84</sup> U. Kubitscheck, R. Schweitzer-Stenner, D. J. Arndt-Jovin, T. M. Jovin, and I. Pecht, *Biophys. J.* **64**, 110 (1993).

studying the binding of haptens to monoclonal immunoglobulins on cell surface receptors.<sup>85</sup>

### Controls

In all of the above techniques it is important to subtract background arising from Raman, specular, or other sources. The best way to do this is to prepare a sample identical to the fluorescence samples, but without the attached dyes. It is also important to assure that the energy transfer is arising only from intramolecular energy transfer, and not from diffusional contact or aggregation. To control for this, one can mix a donor-only labeled sample and an acceptor-only labeled sample under conditions where they will not form a donor-acceptor complex. Noncomplementary DNA strands, for example, can be mixed together, or a donor-only labeled protein can be mixed with an acceptor-only labeled protein, etc. FRET measurements should also be made with magic angle settings (analyzer set to 54°) to assure no polarization artifacts.

### Examples and Application of Fluorescence Resonance Energy Transfer

#### *Human Immunodeficiency Virus Protease*

Matayoshi and co-workers have developed a simple and effective means for measuring HIV protease activity using FRET.<sup>34</sup> This is an example of measuring dynamics with FRET. The idea is to use a relatively short polypeptide that HIV can cleave into two and attach a donor on one end and an acceptor on the other end (see Fig. 5A). If the polypeptide is intact, the donor is highly quenched. On cleavage, the energy transfer is eliminated and the donor fluoresces. By making the polypeptide with an end-to-end distance less than  $R_0$ , the increase in signal on cleavage was 40-fold. They used EDANS as donor and DABCYL, a nonfluorescent dye, as acceptor (see Fig. 5B).

#### *DNA Structure*

One of the more recent applications of FRET is in the study of DNA structure. Clegg and co-workers have published much in this field<sup>16-18</sup> including a 1992 review.<sup>2</sup> They have measured the end-to-end distances of a series of DNA oligonucleotides, ranging from 8 to 20 base pairs (bp)

<sup>85</sup> U. Kubitscheck, M. Kircheis, R. Schweitzer-Stenner, W. Dreybrodt, T. M. Jovin, and I. Pecht, *Biophys. J.* **60**, 307 (1991).

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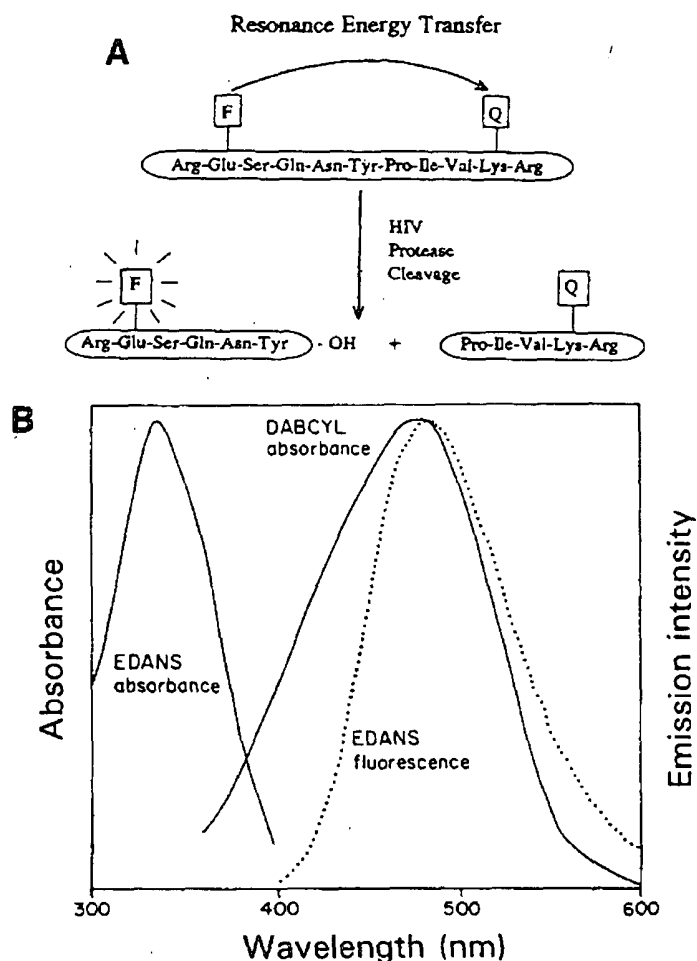


FIG. 5. (A) Use of FRET technique for measuring HIV protease activity. The polypeptide was Arg-Glu(EDANS)-Ser-Gln-Asn-Tyr-Pro-Ile-Val-Lys(DABCYL)-Arg. Because a simple system which could be commercialized was desired (fluorescence means HIV activity, no fluorescence means no HIV activity), the nonfluorescent acceptor was beneficial because it does not contribute signal after cleavage. This system also has the attribute that detailed information about the dyes and their energy transfer properties are not needed. (B) Good overlap between donor emission (maximum at 490 nm, excitation at 340 nm) and acceptor absorbance (maximum of  $28,000 \text{ M}^{-1} \text{ cm}^{-1}$ ). (From Molecular Probes Catalog.<sup>42</sup>)

in length (see Fig. 6). Although the structure of short oligonucleotides is well understood, their detailed study showed conclusively that FRET can be used to study DNA structures, despite some early confusion.<sup>14</sup> The helical repeat of the DNA in solution was observed and the enthalpy of strand hybridization calculated. The helical repeat can be seen in the modulation of the  $R^{-6}$  energy transfer as a function of number of base pairs separating donor and acceptor.



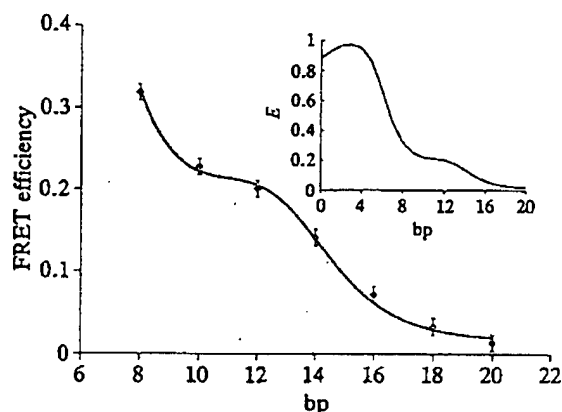


FIG. 6. Fluorescence energy transfer on a series of DNA oligomers differing in length from 8 to 20 bp. The modulation in the energy transfer decay curve (compare with Fig. 2) arises from the helical geometry of the DNA. Inset shows the theoretical FRET signal calculated from Eq. (12) for 0–20 bp. (From Clegg *et al.*<sup>16</sup>)

Clegg and co-workers used fluorescein as donor and tetramethylrhodamine as acceptor ( $R_0 = 45 \text{ \AA}$ ), attaching the dyes to the 5' ends of complementary DNA strands. Others have also used fluorescein–eosin.<sup>14,15</sup> The ability to measure the relatively long distances of a 20-mer (end-to-end distance of approximately  $72 \text{ \AA}$  plus linker lengths) required careful measurement and analysis of the sensitized emission (discussed above in the section on measurement). They took care to ensure that the local environment around the dyes was constant for all samples. They also adjusted the dye linker length to ensure that at least one of the dyes was rotationally mobile. (The fluorescein had a low steady-state anisotropy of 0.07; the rhodamine was less mobile, with an anisotropy of 0.25. In this case, the distance error should be less than 10%.)

Cardullo has also used FRET to study the hybridization of DNA oligonucleotides. Donor (fluorescein) and acceptor (tetramethylrhodamine) were placed on the 5' ends of single-stranded DNA oligomers. On hybridization, energy transfer took place. (This is quite analogous to the HIV study cited above, where here the quantity of interest is hybridization rather than cleavage.) FRET has the advantage that hybridization can be measured at quite low concentration ( $<100 \text{ nM}$ ), in contrast to standard absorbance melting studies which require micromolar quantities. Measurement of hybridization is just a specific example of the more general problem of measuring binding constants. FRET can be useful in this regard because measurements can be made at low concentrations (in contrast to NMR), which is necessary for measuring large binding constants.

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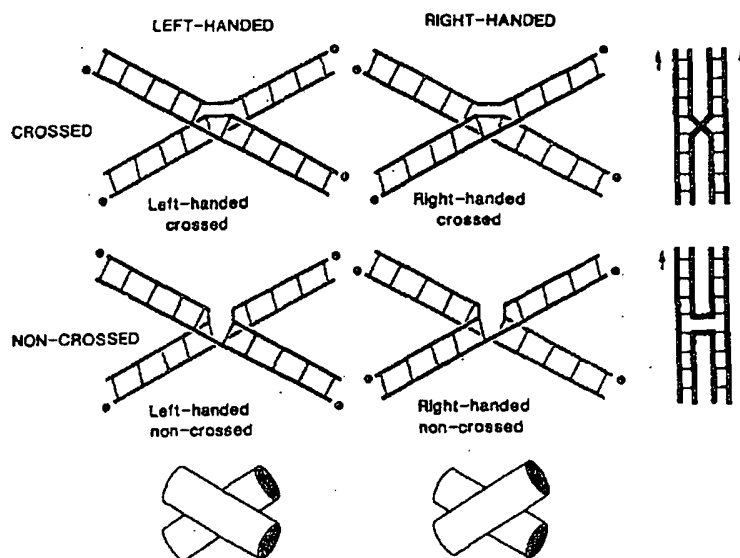


FIG. 7. Use of FRET to show that the overall geometry of the four-way DNA junctions is a right-handed noncrossed structure. The 5' end of the DNA strands are labeled with filled circles. Noncrossed and crossed structures generate antiparallel or parallel alignment of DNA sequences, shown by the arrows at right. The six possible end-to-end distances were measured by labeling the appropriate 5' ends with fluorescein (donor) or tetramethylrhodamine (acceptor) and monitoring energy transfer. (From Murchie *et al.*<sup>17</sup>)

A more sophisticated example of FRET is its application to DNA four-way junctions, also called Holliday junctions. These are believed to be important intermediates in homologous recombination where genetic material is swapped between chromosomes.<sup>86</sup> The geometry of the junction presumably facilitates this swapping process, in combination with enzymes such as resolvase. Lilley and co-workers examined the three-dimensional structure of the junction by several methods, including gel electrophoresis and FRET.<sup>17,18,87</sup> Hagerman and co-workers have also studied the problem.<sup>14,88</sup> Crudely speaking, the junction looks like a nonplanar X (Fig. 7). Gel electrophoresis measurements indicated that the X structure involved strands which did not cross, but this conclusion was based on poorly understood assumptions about DNA mobility in gels. In contrast, the same information could be determined using FRET, where the assumptions are minimal. In addition, the junctions, because they are nonplanar, had a handedness to them (right- or left-handed). FRET could be used to distin-

<sup>86</sup> R. Holliday, *Gen. Res.* **5**, 282 (1964).

<sup>87</sup> D. R. Duckett, A. I. H. Murchie, S. Dickmann, E. von Kitzing, B. Kemper, and D. M. J. Lilley, *Cell (Cambridge, Mass.)* **55**, 79 (1988).

<sup>88</sup> J. P. Cooper and P. Hagerman, *J. Mol. Biol.* **198**, 711 (1987).

guish the crossed from noncrossed structure and right-handed from left-handed because the different proposed structures had different end-to-end distances, which could be measured by end-labeling them with donor and acceptor dyes. Fortunately, measurement of absolute distances was not required to differentiate between the possible structures. Crossed versus noncrossed models, for example, gave different predictions about which two of the six end-to-end distances were closest. Handedness of a junction could be determined by changing the length of one arm of the X (in effect, walking the end-labeled dye around the DNA helix of the arm): left-handed versus right-handed models gave different predictions for when the acceptor and donor would be closest or farthest away from one another. Lilley and co-workers concluded that four-way DNA junctions are right-handed noncrossed structures.<sup>17</sup>

#### *Diffusion-Enhanced Fluorescence Resonance Energy Transfer*

FRET has been used to measure diffusional rates and to examine the accessibility of certain sites to collisional quenching. Stryer *et al.* have presented an excellent review,<sup>8</sup> and Thomas and co-workers put the technique on a firm experimental foundation.<sup>89</sup> Three time regimes can be distinguished in FRET. If  $D$  is the sum of the donor and acceptor diffusion coefficients,  $s$  is the average separation, and  $\tau$  is the donor lifetime, then the static limit, where the donor and acceptor do not appreciably move during the donor lifetime, is when  $6D\tau \ll s^2$ . The intermediate region, which is useful for measuring diffusion constants, is when  $6D\tau \approx s^2$ . The rapid diffusion, where donor and acceptor collide many times and energy transfer is a sensitive function of the closest distance, is when  $6D\tau \gg s^2$ . If the acceptor (or donor) is free to diffuse and has a diffusion constant of approximately  $10^{-6}$  cm<sup>2</sup>/sec (a typical value for a small dye) and the donor has a lifetime of a millisecond, then the rapid diffusion regime can occur if the acceptor concentration is on the order of 1  $\mu$ M or more. The intermediate regime, which is less often used, has been achieved using naphthalene, which has a lifetime of approximately 100 nsec.<sup>90</sup>

To achieve the rapid diffusion limit requires a very long-lived donor. Chelates of terbium, which typically have lifetimes of 1.5–2.2 msec, are frequently used.<sup>8</sup> Fluorescein and rhodamine make excellent acceptors for terbium. Chelates of europium also have long lifetimes, 0.5–2.3 msec,<sup>91</sup> although these appear not to have been used. (Cronce and Horrocks have used europium in a calcium-binding site as a donor in a rapid diffusion

<sup>89</sup> D. D. Thomas, W. F. Carlsen, and L. Stryer, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 5746 (1979).

<sup>90</sup> Y. Elkana, J. Feitelson, and E. Katchalski, *J. Chem. Phys.* **48**, 2399 (1968).

<sup>91</sup> C. C. Bryden and C. N. Reilley, *Anal. Chem.* **54**, 610 (1982).

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experiment.<sup>22</sup> The chelates are often polyaminocarboxylic, such as EDTA. By modifying the chelator to alter its charge and measuring distances of closest approach, one can deduce the local charge surrounding the acceptor. Replacing one or two carboxyl groups of EDTA<sup>4-</sup> with alcohol groups to make HED3A or BED2A, respectively, yields chelates with one or two fewer negative charges. In addition, by attaching an organic chromophore to the chelator, the extremely weak absorbance of the terbium or europium ( $\sim 1 \text{ M}^{-1} \text{ cm}^{-1}$  or less) can be increased several thousandfold.<sup>8,92-94</sup> By operating in D<sub>2</sub>O, the quantum yield of the donor is often assumed to be unity (H<sub>2</sub>O partially quenches lanthanide luminescence<sup>95,96</sup>), although the exact value is difficult to measure.

Energy transfer is typically measured by a decrease in the donor lifetime and, in the rapid diffusion limit, is a sensitive function of closest approach. Energy transfer based on a Förster dipole-dipole mechanism is proportional to  $a^{-4}$  in three dimensions and  $a^{-3}$  in two dimensions (e.g., on membranes), where  $a$  is the distance of closest approach.<sup>8</sup> If the distance of closest approach is less than 10 or 11 Å, energy transfer is dominated by a Dexter type exchange mechanism owing to overlapping wave functions between the donor and acceptor.<sup>8,54</sup> The exact distance of closest approach then becomes difficult to measure.

Lerho *et al.* have used FRET in the rapid diffusion limit to measure the accessibility of H5 histone in chromatin as a function of salt.<sup>30</sup> They labeled the H5 histone with fluorescein-labeled antibody and used TbHED3A and TbEDTA<sup>-</sup> as the freely diffusing donor. They found that the fluorescein becomes less accessible to the chelates as ionic strength increased. In the presence of DNA, they found H5 to be already folded at low ionic strength and the fluorescein inaccessible to the donor chelates. Thomas and co-workers have performed a number of experiments measuring the position of retinal in membranes by its ability to quench freely diffusing terbium chelates.<sup>8,20,97</sup> Retinal is the chromophore in rhodopsin, which acts as a signal transducer in vision, and in bacteriorhodopsin, which acts as a light-driven proton pump. They conclude that retinal in both bacteriorhodopsin and in bovine rhodopsin is buried with respect to both inner and outer membrane surfaces. In the case of rhodopsin, they measure a distance of 22 Å from the inner surface and 28 Å from the outer

<sup>92</sup> A. Canfi, M. P. Bailey, and B. F. Rocks, *Analyst* **114**, 1405 (1989).

<sup>93</sup> T. Ando, T. Yamamoto, N. Kobayashi, and E. Munekata, *Biochim. Biophys. Acta* **1102**, 186 (1992).

<sup>94</sup> A. K. Saha *et al.*, *J. Am. Chem. Soc.* **115**, 11032 (1993).

<sup>95</sup> W. D. Horrocks, Jr., and D. R. Sudnick, *Acc. Chem. Res.* **14**, 384 (1981).

<sup>96</sup> W. D. Horrocks, Jr., and D. R. Sudnick, *J. Am. Chem. Soc.* **101**, 334 (1979).

<sup>97</sup> D. D. Thomas and L. Stryer, *J. Mol. Biol.* **154**, 145 (1982).

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surface<sup>97</sup>; for bacteriorhodopsin they measure the retinal to be approximately 10 Å from the periplasmic surface.

### *Generating New Dyes*

An application of FRET which is still in its infancy but shows great promise is the production of new heterodimeric dyes. One dye serves as the energy donor, the other as the energy acceptor. Over 10 years ago, Glazer and Stryer applied this technique to fluorescent phycobiliproteins,<sup>39</sup> covalently attaching phycoerythrin to allophycocyanin via a disulfide cross-link. The extent of energy transfer was 90%. The complex had the intense absorption of phycoerythrin around 545 nm, with the fluorescence maximum of allophycocyanin (660 nm), a Stokes shift of 115 nm. Glazer and co-workers have applied this technique to DNA dyes.<sup>40,41</sup> Again, the extent of energy transfer is approximately 90%, and they have created a series of dyes which have the same or similar absorption characteristics but with differing emission wavelengths (see Fig. 8). This allows simultaneous excitation with one light source and independent detection of the emission. In addition, the linker used to join the donor and acceptor is positively charged to enhance binding to the DNA and is of such a length to promote intercalation. Because the dyes intercalate and are therefore separated by a DNA base, there does not appear to be excitonic coupling, which would alter the spectral characteristics of the individual dye. The energy transfer mechanism is therefore presumed to be Förster type, and the spectral characteristics of the compound dye are therefore very similar to the sum of those of the individual dyes. Furthermore, a large number of such dyes is possible because the spectral overlap between donor and acceptor need not be large since they are so close together that efficient energy transfer takes place. By choosing one dye (donor) with large absorbance (the quantum yield need not be large) and the other dye (acceptor) with good quantum yield, unusually bright dyes should be possible (see A. Waggoner, this volume [15]). Molecular Probes has generated a series of DNA dyes based on homodimeric and heterodimeric compounds which span a wide wavelength range.<sup>42</sup> Application to molecules other than DNA will depend on the ability to link dyes without creating significant excitonic coupling.

### **Problems and Future Directions: Luminescence Resonance Energy Transfer Using Lanthanide Chelates**

Despite the numerous successful applications of FRET, the technique has a number of drawbacks. First, the maximum distance which can be measured is less than optimal for many biological applications. Second,

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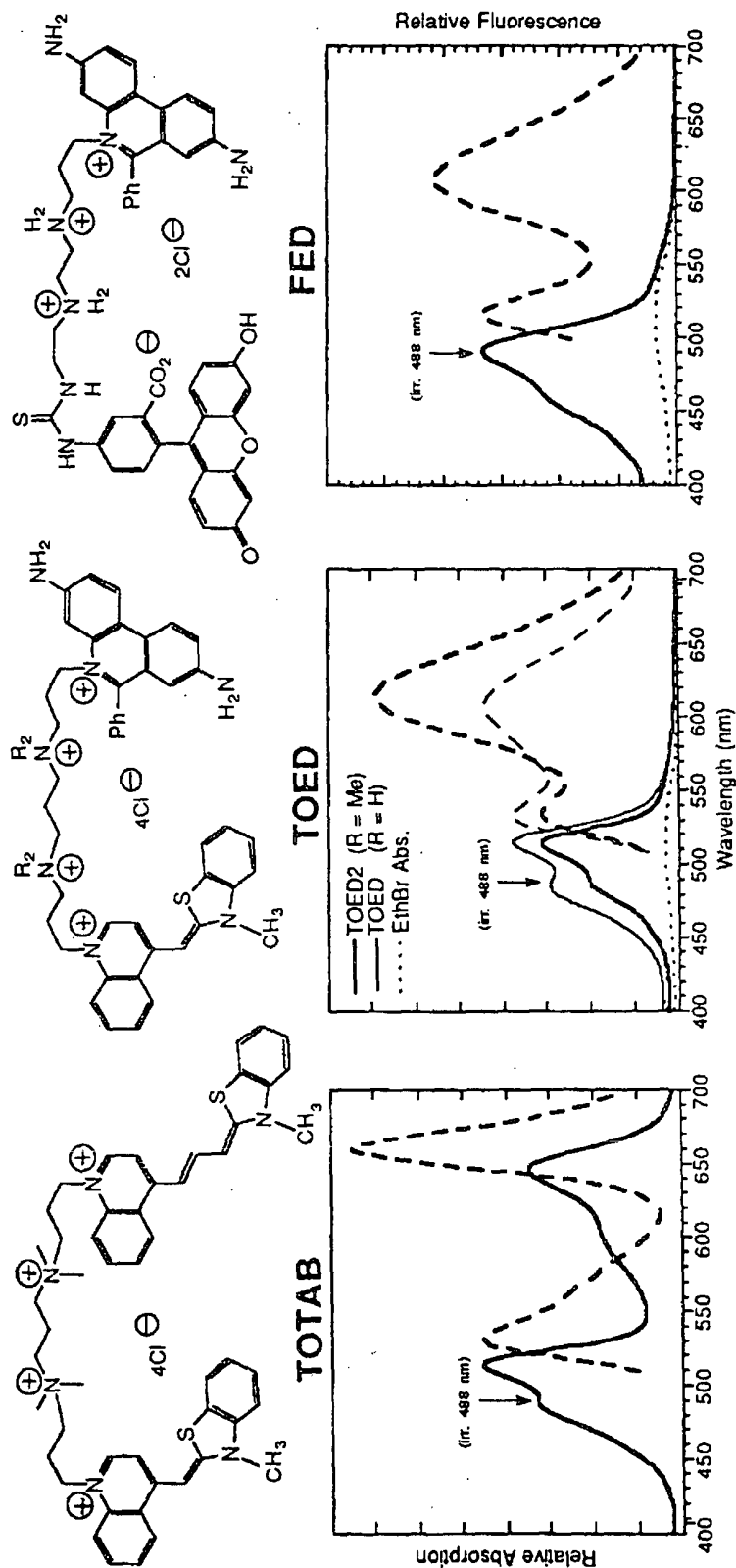


Fig. 8. Use of FRET to generate new DNA dyes. TO stands for thioazole orange, AB for Azure Blue, F for fluorescein, and ED for ethidium bromide. The dyes bind tightly to DNA such that they are stable even during electrophoresis. Solid lines represent absorption; dashed lines, emission. (From Benson *et al.*<sup>40,41</sup>)

the lifetime of commonly used donor fluorophores are short (typically a few nanoseconds) and are often multiexponential, making lifetime measurements difficult and of limited accuracy. An accuracy of 10% limits measurements of quenching of more than 10% and hence less than  $1.45R_0$ . For  $R_0$  values of 40–55 Å, the largest yet attained for small dyes, the maximum measurable distance via donor quenching is therefore 58–72 Å. Third, when measuring the sensitized emission of acceptor the signal-to-background ratio is poor, typically on the order of 1:1. The background arises from interfering fluorescence from the donor and from direct excitation of the acceptor by the laser or excitation light. The poor signal-to-background ratio limits the maximum measurable distance and also makes measurement of the lifetime of the sensitized emission not feasible. The large background also severely inhibits the use of FRET on biological systems that are impure (where, e.g., only a small percentage of donor-acceptor complexes form). Fourth, distances are difficult to determine precisely because of the uncertainty in  $\kappa^2$ .

We have developed an energy transfer system which overcomes these difficulties. We use a luminescent lanthanide chelate as donor and an organic dye such as fluorescein, rhodamine, or CY-5 as acceptor. A number of workers have noted that the luminescent lanthanide elements terbium and europium are attractive donors because they have multiple transition dipole moments such that they act as randomized donors even in the absence of any rotational motion. This limits  $\kappa^2$  ( $1/3 < \kappa^2 < 4/3$ ) even if the acceptor is stationary. Furthermore, the lifetimes are extremely long (0.6–2.3 msec) and single exponential, and thus are easy to measure. The quantum yields of the donors are also likely to be large (approaching 1 in  $D_2O$ ), although the exact value is difficult to measure. The lanthanides can be relatively easily attached to macromolecules via the chelator, and, by covalently coupling an organic chromophore onto the chelator, the lanthanides can also be easily excited.<sup>92–94,98,99</sup> In addition, the spectral overlap is large when using terbium as donor and fluorescein or rhodamine as acceptor, or when using europium as donor and CY-5 as acceptor. The net result is unusually large  $R_0$  values exceeding 50 Å (depending on whether the experiment is performed in  $H_2O$  or  $D_2O$ , what quantum yield is assumed, and which donor-acceptor pair is used). With terbium and rhodamine, for example, we calculate an  $R_0$  of 65 Å, assuming the quantum yield of terbium is 1 in  $D_2O$ , and find that energy transfer experiments are consistent with this value<sup>98</sup> (see below). Using europium and CY-5 in  $D_2O$  we calculate an unusually large  $R_0$  of 70 Å and, again, find that experiments are consistent with this value.<sup>99</sup> Using a europium cryptate

<sup>98</sup> P. R. Selvin and J. E. Hearst, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 10024 (1994).

<sup>99</sup> P. R. Selvin, T. M. Rana, and J. E. Hearst, *J. Am. Chem. Soc.* **116**, 6029 (1994).

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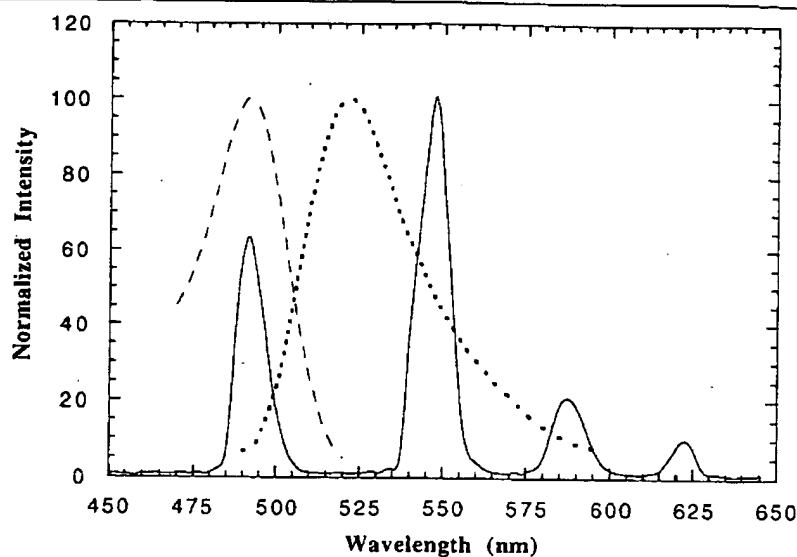


FIG. 9. Emission spectrum of a terbium chelate (structure shown in Fig. 10), used as a donor (solid line), along with absorbance (dotted line) and emission (dashed line) spectra of fluorescein, used as acceptor. Note that emission of the donor is silent around 520 nm, where the acceptor emission is maximal. Also note the excellent overlap between the 492 nm donor emission line and fluorescein absorbance, leading to a large  $R_0$  ( $\approx 52\text{\AA}$ ). By using a pulsed excitation source and monitoring at 520 nm, any signal arises only from sensitized emission, that is, fluorescein fluorescence due only to energy transfer (see Fig. 11).

as donor and allophycocyanin as acceptor, Mathis reports an exceptionally large  $R_0$  of  $90\text{\AA}$ .<sup>100</sup>

For all of these reasons, lanthanide donors and organic acceptors have been used in diffusion-enhanced energy transfer experiments. We find, however, that they can be used quite effectively in static FRET as well, as long as care is taken to ensure that no intermolecular energy transfer takes place. Furthermore, the most powerful aspect of these donor-acceptor pairs appears not to have been recognized until relatively recently: one can measure the sensitized emission of the acceptor without any interfering background.<sup>98,99,100</sup> This is in contrast to most donor-acceptor pairs, where the sensitized emission is much less than the background. Such a dark-background sensitized emission experiment has a number of advantages. First, like all sensitized emission experiments, it is less susceptible to artifacts than donor quenching. Second, because even a small amount of energy transfer yields fluorescence much above background, distances well beyond  $R_0$  can be measured. Third, it is possible to measure the lifetime of the sensitized emission. (We measure not the nanosecond lifetime of each acceptor molecule, but the millisecond decay of the ensemble of acceptors.) By measuring the lifetime of the sensitized emission, studies are insensitive to concentration effects, to quantum

<sup>100</sup> G. Mathis, *Clin. Chem.* **39**, 1953 (1993).



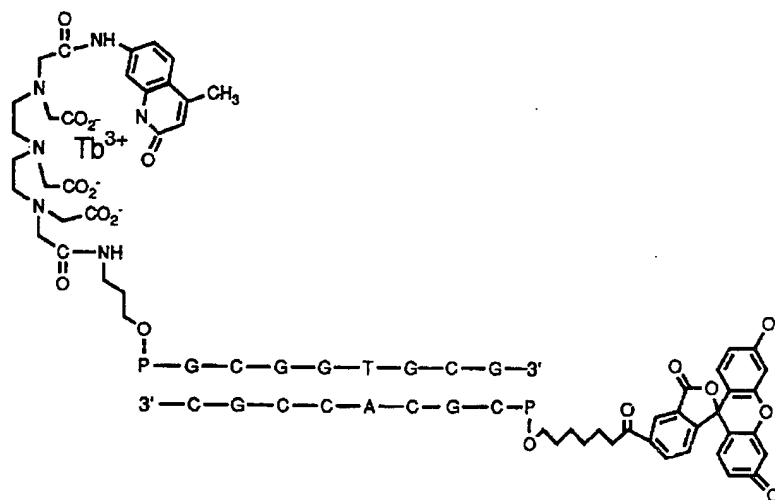


FIG. 10. Structure of terbium chelate, namely, terbium diethylenetriaminepentaacetic acid coupled to carbostyryl 124. The carbostyryl absorbs light (maximally at 327 nm) and transfers energy to the terbium. The net result is an increase in the effective absorbance of the terbium by several thousandfold. The DTPA chelate shields the terbium from the quenching effects of water and allows for easy attachment to macromolecules. Here the macromolecule is an 8-mer DNA oligomer modified with a primary amine on the 5' end. The acceptor, fluorescein, is attached to the 5' end of a complementary DNA oligomer.

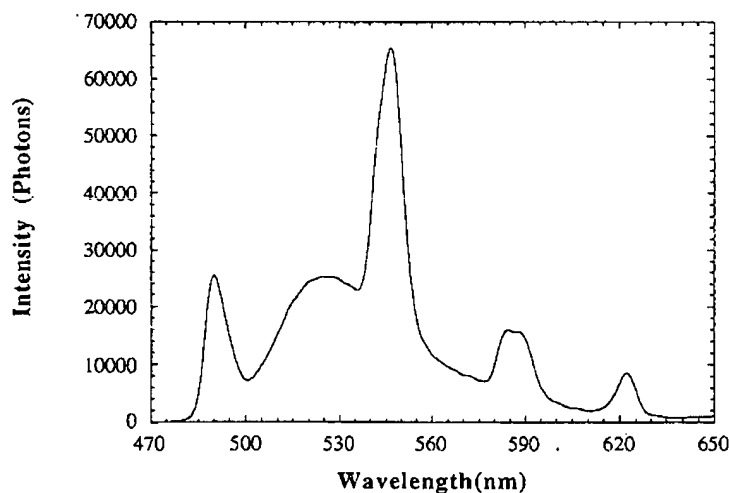


FIG. 11. Emission spectrum showing energy transfer after excitation at 337 nm with a 2 nsec pulse, with the signal collected after a 80  $\mu$ sec delay with a 7 msec gate. Each point (taken every 2 nm) is the average of 160 pulses. The sensitized emission signal-to-background ratio at 520 nm is approximately 400:1. The efficiency of energy transfer is 70%.

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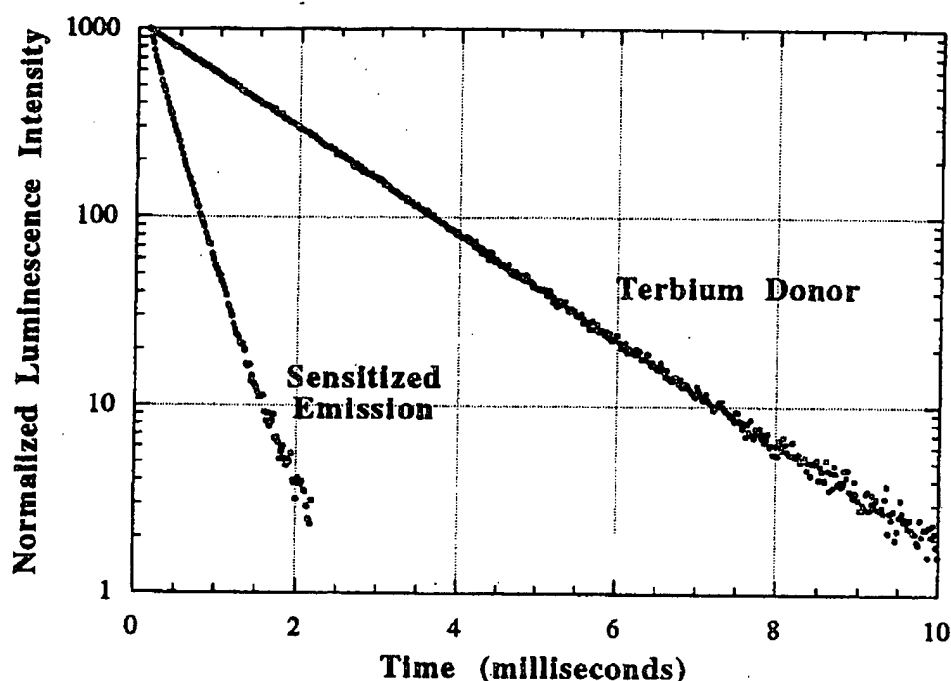


FIG. 12. Lifetime of 1.56 msec for unquenched terbium donor and 270  $\mu$ sec for the fluorescein sensitized emission of donor-acceptor complex from Fig. 11. The sensitized emission lifetime indicates 83% quenching. Only completely labeled donor-acceptor complexes contribute to the sensitized emission signal. The somewhat higher energy transfer efficiency measured via lifetime (83%) versus intensities (70%, Fig. 11) is due to some donor-only species which contributes signal to intensity but not lifetime measurements.

yields (except as they affect  $R_0$ ), and to incomplete labeling; only those species that are labeled with both donor and acceptor contribute to the signal.

We achieve a dark-background, sensitized emission experiment by eliminating the two usual sources of background. The donor fluorescence can be eliminated because the lanthanide luminescence is highly spiked and has regions of darkness (see Fig. 9). Terbium, for example, is silent at 520 nm. The fluorescence of the acceptor arising from direct excitation can be eliminated by using pulsed excitation and gating the detector off for a brief period, during which time the acceptor fluorescence dies away (lifetime typically a few nanoseconds) while the donor remains excited (lifetime of 1.5 msec in  $H_2O$ , 2.2 msec in  $D_2O$  for terbium). As a result, any fluorescence striking the detector in the donor dark region after a few microseconds is due only to energy transfer.

In Figures 11 and 12 we show an experiment where a terbium chelate (terbium diethylenetriaminepentaacetic acid coupled to carbostyryl 124) transfers energy to fluorescein. The donor and acceptor are separated

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by an 8-mer DNA duplex oligomer (Fig. 10). The sensitized emission is measured with no background at around 520 nm (Fig. 11).

The 8-mer DNA oligomer is used to separate rigidly the donor and acceptor, and the complex is immersed in a viscous sucrose solution to eliminate intermolecular interactions. (We find that intermolecular interactions do not significantly contribute to energy transfer for this system even without the sucrose, presumably because of charge repulsion of the DNA oligomers and because the diffusional rate of energy transfer is much smaller than that energy transfer arising from the acceptor fixed to the same DNA oligomer as the donor.) The efficiency of energy transfer based on both donor quenching and the integrated sensitized emission area is 70%. Figure 12 shows measurement of the unquenched donor lifetime (1.5 msec) in the absence of acceptor and the sensitized emission lifetime in the donor-acceptor complex (lifetime 270  $\mu$ sec). The lifetimes indicate a quenching of 83%. (The discrepancy between 70 and 83% appears to be due to a small fraction of terbiums which cannot transfer energy, presumably arising from some donor-only complex. This lessens the percent quenching as measured by donor intensity quenching but does not affect the percent quenching as measured by the sensitized emission lifetime.) Note that the sensitized emission lifetime measurement is completely insensitive to incomplete labeling and to absolute concentrations.

The ability to measure intensities and lifetimes of both donor and acceptor emission with high accuracy and excellent signal-to-background, coupled with the unusually large  $R_0$ s, makes luminescence resonance energy transfer a potentially powerful technique for measuring distances in biological systems.

#### Acknowledgments

P.R.S. acknowledges support from the National Institutes of Science, grant RO1GM41911, and from the Office of Energy Research, Office of Health and Environmental Research of the Department of Energy under contract DEACO3-76SF00098.

### [14] Time-Resolved Fluorescence Spectroscopy

By ALFRED R. HOLZWARTH

#### Introduction

Fluorescence, that is, the phenomenon of light emission from an electronically excited state of a molecule, has found numerous and still rapidly growing applications for studies in the life sciences. The principal advan-

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## REVIEW

Resonance Energy Transfer: Methods and Applications<sup>1</sup>Pengguang Wu and Ludwig Brand<sup>2</sup>*Department of Biology and The McCollum-Pratt Institute,  
The Johns Hopkins University, Baltimore, Maryland 21218*

Resonance energy transfer is widely used in studies of biomolecular structure and dynamics. It provides information about distances on the order of 10 to 100 Å and is thus suitable for investigating spatial relationships of interest in biochemistry. The information available from energy transfer studies has been enhanced by the advances in instrumental methods and procedures of data analysis related to fluorescence decay studies. Some practical aspects of the method are reviewed. These include sample preparation, Förster type distance determination, methods of detecting energy transfer and calculating transfer efficiency, time-resolved measurements, and data analysis. Applications of resonance energy transfer, including qualitative measurements as well as microscopy, average distance estimation, and distance distribution analysis, are surveyed. © 1994 Academic Press, Inc.

Structural and dynamic information of macromolecular assemblies is clearly important for understanding their functions. Resonance energy transfer as measured by steady-state and time-resolved fluorescence can provide important information in this regard and thus is a phenomenon of interest in analytical biochemistry. Distance information from 10 to 100 Å can be obtained, which makes the method well suited for studying many biological systems (1-9). Although the phenomenon of resonance energy transfer was observed by Perrin at the beginning of the century, it was Förster who proposed a theory describing long-range molecular interactions by resonance energy transfer in the late 1940's and derived a transfer rate equation that relates to the interchromophore distance and the spectroscopic properties of the

chromophores. The theory has been verified experimentally by Stryer and co-workers (3). The dependence of transfer rate on the sixth power of distance and on the overlap integral was shown to hold. Thus resonance energy transfer can be used as a spectroscopic ruler for long-range distance determination in biology (3). In many applications the flexibility of the probe linker arm provides enough dynamic averaging such that orientation factor does not significantly affect the accuracy of average distance measurements. The uncertainty due to orientation factor has nonetheless been a major negative factor in using this technique and several methods were proposed to reduce it (10-13). Recently, the problem was clarified and proven to be solvable in that one can actually utilize apparent distance distribution measurements to reduce the large uncertainty (14). Thus average distances can be reliably obtained from resonance energy transfer measurements.

There are several distinctive aspects regarding resonance energy transfer studies. (a) Measurement of energy transfer is based on fluorescence detection, thus ensuring high sensitivity. In addition to data acquisition with a conventional spectrophotofluorometer, energy transfer methods can be incorporated into liquid chromatography assays, electrophoresis, microscopy, *in vivo* detections, and flow cytometry, to mention only a few. (b) Structural information, which is otherwise difficult to gather, can be obtained, albeit at low resolution, regardless of the complexity or heterogeneity of the system. (c) Since the time scale of resonance energy transfer is on the order of nanoseconds, many processes (such as slow conversion of conformers) that are time-averaged in other techniques can be resolved.

Although numerous reviews on resonance energy transfer are available (1-8), there is a paucity of information regarding the practical aspects of the method. Application of the method requires some working knowledge in areas including biochemistry (sample isolation and purification), chemistry (chemical modification

<sup>1</sup> Supported by NIH Grant GM-11632.

<sup>2</sup> To whom correspondence should be addressed. Fax: (410) 516-5213.

with suitable fluorophores), physical measurements (steady-state or time-resolved), and data analysis (usually nonlinear least squares). With recent advances in molecular biology, purification techniques, instrumentation, and computer data analysis, many obstacles associated with effective applications of resonance energy transfer method to biological problems have been eliminated or reduced. It is the purpose of this review to bring together some of the experimental aspects of the method, including Förster distance determination, sample preparation, energy transfer efficiency measurements by steady-state and time-resolved fluorescence, and data analysis.

## FACTORS AFFECTING ENERGY TRANSFER RATES

Energy transfer by dipolar interaction is described by the Förster rate equation (9)

$$k = \frac{1}{\tau_D} \left( \frac{R_0}{r} \right)^6, \quad R_0^6 = 8.785 \times 10^{-5} \frac{\kappa^2 \phi_D J}{n^4}, \quad [1]$$

where  $\tau_D$  is the donor lifetime in the absence of acceptor,  $r$  is the donor-acceptor distance, and  $R_0$  is the Förster or critical transfer distance at which the energy transfer rate is equal to the decay rate. The Förster distance is related to the orientation factor,  $\kappa^2$  between donor and acceptor, and the spectroscopic properties of the donor and acceptor.  $\phi_D$  is the quantum yield of the donor in the absence of acceptor. The index of refraction,  $n$ , is generally taken to be 1.4 in aqueous solution.  $J$  is the overlap integral between the donor and acceptor,

$$J = \int F_D(\lambda) \epsilon_A(\lambda) \lambda^4 d\lambda, \quad [2]$$

where  $F_D$  is the peak-normalized fluorescence spectrum of the donor.  $\epsilon_A$  is the molar absorption coefficient of the acceptor. The scaling constant is set such that when  $\epsilon$  is in units of  $M^{-1} cm^{-1}$  and wavelength  $\lambda$  in units of nm, the Förster distance is in units of Å. Procedures for evaluating  $J$  and  $R_0$  are shown in the next section.

Factors influencing energy transfer rates have been discussed extensively in reviews (1-8). Experiments with model systems have shown that Förster theory is valid and applicable to distance determination. The orientation factor remained controversial until recently. The uncertainty from the orientation factor in average distance estimation originates from the fact that all distance calculations in resonance energy transfer measurements are referenced to Förster distance, which is calculated from spectroscopic properties of the donor and acceptor. There is no internal distance reference involved. If a donor-acceptor pair adopts a particular

orientation such that their orientation factor is far off from the dynamically averaged value  $\frac{2}{3}$ , then the calculated distance is also off from the actual one when  $\langle \kappa^2 \rangle = \frac{2}{3}$  is used. Several approaches have been proposed to reduce the uncertainties. These include anisotropy measurements to obtain the limits of the orientation factor (10), the use of multiple pairs of donor-acceptor (11), or the statistical interpretation of the results (12). Selection of chromophores with multiple electronic transitions also helps reduce uncertainties associated with the orientation factor (13). Still, the issue of orientation factor has continued to fill discussions in numerous papers in the literature. The problem was approached recently from the viewpoint of distribution (14) in time-resolved fluorescence. Quenching of donor fluorescence is due to acceptors at different distances as well as orientations, and due to motions of donor or acceptor. The superposition of all these contributions leads to a donor decay that can be analyzed by an apparent distance distribution. It is the distribution of donor-acceptor distances and their respective orientations, not the limits of orientation, that determine the observed average distance. Since the apparent distance distribution can be obtained experimentally, the uncertainties due to orientation factor can be assessed to a reasonable degree of accuracy. As long as there is some distribution and donor and acceptor are not too close (relative to  $R_0$ ), their average distance should be reliably obtained. In the rapid diffusion limit, as in the case of long-lived luminescence of metal ions and triplet state decays, orientational contribution is completely averaged. Dependence of energy transfer rate on various geometries in this limit have been analyzed (15,16).

Förster theory is based on very weak interactions between donor and acceptor (9). Thus spectroscopic properties such as absorption of one chromophore should not be altered by direct interaction in the presence of another. This defines the shortest distance range over which the theory is valid. How close it may be depends on donor-acceptor pairs. For some donor-acceptor pairs Förster theory is valid below 10 Å as determined by subpicosecond techniques (17). When donor and acceptor are too close other interaction or energy transfer such as the Dexter mechanism (18) may take place, thus complicating distance evaluations. One factor that has sometimes been overlooked is that the spectroscopic properties of chromophores such as quantum yield or spectral shape may change in different environments, leading to results that appear to question the validity of Förster theory. In the case of dye binding to nucleic acids, complications include nonrandom binding (or clustering) or stacking interactions. Förster theory was questioned in energy transfer between identical molecules bound to DNA (19) which was shown later to be valid when bound molecules were not close enough for

clustering to contribute (20). Similarly, problems associated with application of Förster theory to oligonucleotides (21) may be overcome with appropriate analysis of the data (22).

### FÖRSTER DISTANCE DETERMINATION

Energy transfer measurement is most sensitive to distance variation when donor-acceptor separation is close to their Förster distance. Thus, the approximate dimension of the system to be studied is the most important factor to be considered when choosing a dye pair. In steady-state measurements, other considerations include such factors as high quantum yield and stability against hydrolysis and photolysis. For distance distribution analysis in time-resolved experiments, chromophore decay lifetimes and length of linker arms impose further constraints. Since there is no internal distance reference in resonance energy transfer, all distances calculated from transfer efficiencies are relative to a Förster distance evaluated from spectroscopic properties of donor and acceptor. Förster distance thus provides a reference point in distance measurements.

Calculation of Förster distance within a donor-acceptor pair requires the following: (a) donor quantum yield, (b) fluorescence emission spectrum of the donor, and (c) the molar absorption coefficient of the acceptor. The emission spectrum of the donor,  $I_{\text{obs}}(\lambda)$ , from a fluorescence spectrophotometer generally contains a wavelength-dependent instrument response  $S(\lambda)$  such that  $I_{\text{obs}}(\lambda) \propto S(\lambda)I(\lambda)$ , where  $I(\lambda)$  is the true spectrum of donor. On some instruments manufacturers provide instrument response correction. When this is not available, a reference compound with known spectrum,  $I^{\text{ref}}(\lambda)$ , can be used to obtain  $S(\lambda)$ . In this case for the reference,  $I_{\text{obs}}^{\text{ref}}(\lambda) \propto S(\lambda)I^{\text{ref}}(\lambda)$ , and the sample of interest,  $I_{\text{obs}}(\lambda) \propto S(\lambda)I(\lambda)$ . One has  $I(\lambda) (=F(\lambda)) = I_{\text{obs}}(\lambda)I^{\text{ref}}(\lambda)/I_{\text{obs}}^{\text{ref}}(\lambda)$  at each wavelength.  $I(\lambda)$  can then be used in overlap integral evaluation which is a simple summation,  $J = \sum_i F_D(\lambda_i)\epsilon_A(\lambda_i)\lambda_i^4/\sum_i F_D(\lambda_i)$ , in units of  $\text{M}^{-1}\text{cm}^{-1}\text{nm}^4$  (see Eq. [2]). This can be evaluated using computer programs, spread sheets, or mathematical packages.

Similarly, quantum yield,  $\phi$ , of donor can be obtained with reference to a compound of known quantum yield,  $\phi_{\text{ref}}$ . The total corrected intensity (summed over all emission wavelengths) is  $I \propto \phi A$ , where  $A$  is the absorbance at the excitation wavelength. One can either use a single measurement to obtain  $\phi = I\phi_{\text{ref}}A_{\text{ref}}/(AI_{\text{ref}})$  or use a series of absorbances ( $A < 0.1$ ) of each, obtain the slope of  $I$  vs  $A$ , and calculate  $\phi$ . When sample and reference solvents are very different, a correction due to difference in indices of refraction needs to be performed ( $\phi/n^2 = \phi_{\text{ref}}/n_{\text{ref}}^2$ , where  $n$  and  $n_{\text{ref}}$  are the refractive indices of the solvents containing the sample of interest and

the reference compound, respectively). Lists of compounds that can serve as references in quantum yield determination and instrument response correction are available (23–28). Between 400 and 600 nm, quinine in 1 N  $\text{H}_2\text{SO}_4$  with  $\phi = 0.55$ , or fluorescein in 0.1 N NaOH with  $\phi = 0.92$ , can be used. Between 300 and 450 nm, tryptophan (24,29,30) with  $\phi = 0.14$  in water at 25°C, or 2-aminopyridine in 0.1 N  $\text{H}_2\text{SO}_4$  with  $\phi = 0.6$  (31,32), can be used.

Table 1 shows a partial list of Förster distances of donor-acceptor pairs that have been applied to biological samples. They are used as a general guide when selecting a particular pair. A comprehensive list of Förster distances of various compounds can be found elsewhere (77). Since quantum yield and spectral shape may be environment sensitive, Förster distances may vary as solution condition changes. Reported Förster distances range from 10 to 70 Å. Thus average distances up to 120 Å can be measured. The highest Förster distance reported for a single donor-acceptor pair is 80 Å for the rhodamine B-malachite green (78). A further extension of Förster distance may be achieved by an acceptor molecule comprising clusters of acceptors with high molar absorption coefficient for each acceptor.

In proteins tryptophan or tyrosine sometimes may be used as chromophores in distance measurements. Förster distances using tryptophan as a donor is listed in Table 2. In most cases, the Förster distance is limited to about 30 Å.

### SAMPLE PREPARATION

Measurements of resonance energy transfer require a donor and an acceptor chromophore. Few intrinsic donor-acceptor pairs are available and thus extrinsic probes are often used. Noncovalent or random modification of samples involve many steps beyond the scope of this review. We are primarily concerned with unique and covalent modifications here. Sample preparation generally involves obtaining pure and homogeneous sample, characterization of the functional group to be modified, carrying out modification and purification, and reconstitution to the system interested. A well-prepared and characterized sample can save a lot of time and effort in measurements and data interpretation.

*Proteins and peptides.* Many references can be found on modification of various groups in proteins or peptides. These are summarized in reviews or monographs (4,87–92). Although several groups may be used to couple to a chromophore, the thiol group is perhaps the best candidate in that many reactions are either thiol specific or selective, and thus unique labeling is possible. With site-directed mutagenesis a thiol group can be added to or deleted from a desired position. In



TABLE 1  
 Förster Distances

Donor	Acceptor	$R_0$ (Å)	References	Donor	Acceptor	$R_0$ (Å)	References
Naphthalene	Dansyl	22	33	IAEDANS	TNP-ATP	40	60
IANBD	DDPM	25	34	$\epsilon$ -A	IANBD	40	61
IAEDANS	DDPM	25-29	35, 36	NBD	SRH	40-74	62
DNSM	LY	26-32	37	ISA	TNP	42	63
IAEDANS	IANBD	27-51	38, 39	Dansyl	ODR	43	64
$\epsilon$ -A	F <sub>2</sub> DNB	29	40	DANZ	IAF	44-49	65
Pyrene	Bimane	30	41	FNAI	EITC	45	42
ANAI	IPM	30	42	NBD	LRH	45-70	62
IAANS	IAF	31	43	IAF	EIA	46	54
$\epsilon$ -A	F <sub>2</sub> DPS	31	40	FITC	ENAI	46	42
$\epsilon$ -A	DDPM	31	44	Proflavin	ETSC	46	66
IAEDANS	TNP	31-40	45, 46	CPM	TNP-ATP	46	48
MNA	DACM	32	47	IAEDANS	IAF	46-56	38, 43
PM	NBD	32	48	CPM	Fluorescein	47	67
FITC	TNP-ATP	32	49	IAEDANS	FITC	49	68, 69
DANZ	DABM	34	50	FITC	TMR	49-54	70, 71
NCP	CPM	34	51	IAF	TMR	50	53
NAA	DNP	33-37	52	CF	TR	51	72
LY	TNP-ATP	35	37	CPM	FTS	51	73
IAF	diI-C <sub>18</sub>	35	53	$\epsilon$ -A	TNP-ATP	51	46
IAF	TMR	37	54	CPM	FM	52	73
FMA	FMA	37	55	LY	EM	53	74
PM	DMAMS	38	56	FITC	EITC	54	75
mBBR	FITC	38	57	IAEDANS	DiO-C <sub>14</sub>	57	53
mBBR	DABM	38	58	IAF	ErITC	58	49
$\epsilon$ -A	NBD	38	59	FITC	EM	60	71
Pyrene	Coumarin	39	41	FITC	ETSC	61-64	66
IPM	FNAI	39	42	FITC	ErITC	62	49
IAEDANS	DABM	40	60	BPE	CY5	72	76

Note. ANAI, 2-anthracene *N*-acetylimidazole; BPE, B-phycoerythrin; CF, carboxyfluorescein succinimidyl ester; CPM, 7-diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin; CY5, carboxymethylindocyanine-*N*-hydroxysuccinimidyl ester; diI-C<sub>18</sub>, 1,1'-dioctadecyl-3,3',3'-tetramethyl-indocarbocyanine; diO-C<sub>14</sub>, 3,3'-ditetradecyloxycarbocyanine; DABM, 4-dimethylaminophenylazo-phenyl-4'-maleimide; DACM, (7-(dimethylamino)coumarin-4-yl)-acetyl; DANZ, dansylaziridine; DDPM, *N*-(4-dimethylamino-3,5-dinitrophenyl)maleimide; DMAMS, dimethylamino-4-maleimidostilbene; DMSM, *N*-(2,5-dimethoxystiben-4-yl)-maleimide; DNP, 2,4-dinitrophenyl;  $\epsilon$ -A, 1,N<sup>6</sup>-ethenoadenosine; EIA, 5-(iodoacetamidoeosin; EITC, eosin-5-isothiocyanate; ENAI, eosin *N*-acetylimidazole; EM, eosin maleimide; ErITC, erythrosin-5'-isothiocyanate; ETSC, eosin thiosemicarbazide; F<sub>2</sub>DNB, 1,5-difluoro-2,4'-dinitrobenzene; F<sub>2</sub>DPS, 4,4'-difluoro-3,3'-dinitrophenylsulfone; FITC, fluorescein-5-isothiocyanate; FM, fluorescein-5-maleimide; FMA, fluorescein mercuric acetate; FNAI, fluorescein *N*-acetylimidazole; FTS, fluorescein thiosemicarbazide; IAANS, 2-(4'-iodoacetamido)anilino)naphthalene-6-sulfonic acid; IAEDANS, 5-(2-((iodoacetyl)amino)ethyl)amino)-naphthlene-1-sulfoni acid; IAF, 5-iodoacetamidofluorescein; IANBD, *N*-((2-(iodoacetoxy)ethyl)-*N*-methyl)amino-7-nitrobenz-2-oxa-1,3-diazole; IPM, 3(4-isothiocyanatophenyl)7-diethyl-4-amino-4-methylcoumarin; ISA, 4-(iodoacetamido)salicylic acid; LRH, lissaminerhodamine; LY, Lucifer yellow; mBBR, monobromobimane; MNA, (2-methoxy-1-naphthyl)-methyl; NAA, 2-naphthoxyacetic acid; NBD, 7-nitro-2,1,3-benzoxadiazol-4-yl; NCP, *N*-cyclohexyl-*N'*-(1-pyrenyl)carbodiimide; ODR, octadecylrhodamine; PM, *N*-(1-pyrene)-maleimide; SRH, sulforhodamine; TMR, tetramethylrhodamine; TNP, trinitrophenyl; TR, Texas red.

addition, the properties of thiol groups are well studied (93-95). The modification of a thiol group can be reversible or irreversible. Haloacetyl and maleimide labeling reagents lead to irreversible modifications and thus produce more stable products. Chromophores with disulfide bonds give reversible modifications which can be reversed by adding excessive thiol-containing reagents. The donor molecule is always a fluorescent (or luminescent) one for detection. The acceptor molecule can be either fluorescent or nonfluorescent and thus a larger pool of selections exists. For proteins with multiple thiol

groups, different thiols may exhibit different reactivity and selective labeling of one thiol group may be achieved (96).

Conditions of modification vary to a great degree. For oligopeptides or proteins capable of reversible denaturation, harsh conditions may be employed for reactions to take place effectively. For proteins not reversible in denaturation, near native solution conditions dictate labeling reactions.

*Nucleic acids and carbohydrates.* Not many selective groups are available for coupling of chromophores to

TABLE 2  
Förster Distances Using Trp as a Donor

Donor	Acceptor	$R_0$ (Å)	References
Trp	Ru(III)(NH <sub>3</sub> ) <sub>5</sub>	12-16	79
Trp	Nitrobenzoyl	16	80
Trp	Dansyl	21	2
Trp	IAEDANS	22	11
Trp	ANS	23	81
Trp	Anthroyloxy	24	80
Trp	TNB	24	14
Trp	Anthroyl	25	82
Trp	Tyr-NO <sub>2</sub>	26	83
Trp	Pyrene	28	84
Trp	Heme	29	85
Trp	NBS	30	80
Trp	DNBS	33	80
Trp	DPH	40	86

Note. NBS, nitrobenzenesulfonyl; DNBS, dinitrobenzenesulfonyl.

natural bases in nucleic acids or to sugar chains (except the reducing end) in carbohydrates. Reactive groups such as aldehyde, amine, thiol, or phosphothioate can be introduced by analogs or chemical modification (88,97-99).

Important issues concerning sample preparations are purity, specificity of the labeling reaction, uniqueness of labeling site, stability of labels, completeness of labeling reaction, and the potential of alteration in the stability and function of the macromolecule. The concern with purity cannot be overemphasized since background or impurity fluorescence frequently interferes with measurements of energy transfer and impurities can be introduced in many of the sample handling steps. Proper choice of labeling reagents can minimize the problem of stability and selectivity of labeling sites.

If a labeling reaction goes to 100%, which may be the case for some reactions under favorable conditions, the separation of labeled sample from the unreacted reagents is straightforward. On the other hand, for an incomplete reaction, the separation of labeled from unlabeled sample is not a clear-cut problem. The introduction of an aromatic chromophore may change the charge or hydrophobic character of the sample. One may choose reverse-phase or ionic exchange chromatographies to purify labeled samples. If a single thiol group is modified, one may use thiol affinity chromatography. The biggest challenge is probably the double-labeling of a single molecule or subunit. Unique labeling and separation pose many technical difficulties. Nonetheless successful applications can be found (47,52,98). In many applications the fraction of labeling can be measured and energy transfer efficiency corrected without the burden of separation of labeled from unlabeled sample.

## DETECTION OF ENERGY TRANSFER

**Donor quenching.** This is probably the most commonly used method. Excitation is set at the wavelength of donor absorption and the emission of donor is monitored. The emission wavelength of donor is selected such that no contribution from acceptor fluorescence is observed. The presence of acceptor quenches donor fluorescence. Energy transfer efficiency,  $E$ , is calculated from  $E = 1 - I_{DA}/I_D$ , where  $I_{DA}$  and  $I_D$  are donor intensities in the presence and absence of acceptor. Both are normalized to the same donor concentration. It may be difficult to quantitate donor concentration in the presence of acceptor. This problem can be eliminated by time-resolved measurements in which donor concentration is not required,  $E = 1 - \langle\tau_{DA}\rangle/\langle\tau_D\rangle$ , where  $\langle\tau_{DA}\rangle$  and  $\langle\tau_D\rangle$  are amplitude-averaged lifetimes (see below) of donor in the presence and absence of acceptor. The concentration requirement can also be eliminated in steady-state measurements by the method of Epe *et al.* (100). In this approach, enzymatic digestion is performed on labeled samples to obtain an internal concentration reference. Another way of calculating energy transfer efficiency was proposed by Flamion *et al.* (101), in which absorption and emission spectra of donor and acceptor alone are used as basis spectra to obtain the labeling ratio, concentration, and transfer efficiency by a nonlinear least-squares method. Utilization of the entire emission spectral range of donor or acceptor by curve fitting (22) can improve the accuracy of transfer efficiency calculation when donor and acceptor emissions overlap. This can be achieved since there is no change in spectral shape in either donor or acceptor (a condition on which Förster theory is based) and many computer numerical methods are available (102). Once the transfer efficiency is known (with correction for the labeling ratio if it is not 100%), the average distance between donor and acceptor is calculated from  $\bar{r} = R_0(1/E - 1)^{1/6}$ .

Noncovalent binding or association presents some special problems if the binding constant is not high enough. For example a donor is covalently attached to a large molecule, either extrinsic or intrinsic, and an acceptor functions as a ligand analog or an inhibitor. Three issues may be of interest. (a) The binding of ligand may change the fluorescence properties of donor not through direct interaction but through conformational changes of the large molecule. This needs to be taken into account for the transfer efficiency calculation. (b) Addition of acceptor may cause inner filter effect at the excitation wavelength of donor. This leads to an apparent decrease in donor intensity and can be corrected approximately if the absorbance is measured. (c) Intermolecular energy transfer may occur in addition to intramolecular transfer within the macromolecule-ligand complex. This will only be a problem if

acceptor concentration is very high. The critical concentration of acceptor is  $[A_0] = 447/R_0^3$ , where  $R_0$  is in units of Å. The intermolecular transfer efficiency (103) at this concentration is 76%. Diffusion can further enhance energy transfer (104,105). Concentration of acceptor to avoid intermolecular transfer should be much lower than  $447/R_0^3$ . For example, if  $R_0 = 25$  Å then  $[A] = 0.028$  M. With a diffusion coefficient of  $1 \times 10^5$  cm<sup>2</sup> s<sup>-1</sup> and an acceptor concentration of  $1 \times 10^{-4}$  M, there is about 2% intermolecular energy transfer. Thus when acceptor concentration exceeds one hundredth of its critical concentration, possible intermolecular energy transfer should be considered. In some cases, extrapolation of transfer efficiency (38,74) has been used for associating systems.

**Acceptor enhancement.** If an acceptor is fluorescent its fluorescence intensity is enhanced when energy transfer occurs (with excitation into the donor). This is another means to visualize energy transfer from a fluorescence spectrum. In an emission spectrum, one excites at the wavelength of donor absorption and observes the intensity increase of acceptor. In an excitation spectrum, one sets detection at the acceptor emission wavelength and observes enhancements of intensity at a wavelength range where donor absorbs. In most cases acceptors have some absorbances at excitation wavelengths of donor and the calculation of energy transfer efficiency is thus slightly complicated.

**Anisotropy.** When energy transfer occurs between the same molecules in identical environments, fluorescence intensity or lifetime does not change. The anisotropy on the other hand may change due to likely change in chromophore orientation. Many proteins contain multiple tryptophans or tyrosines (106) and energy transfer may occur from one chromophore to another. Due to the complex nature of tryptophan photophysics (107), however, utilization of this phenomenon is difficult. An example of energy transfer between identical molecules is ethidium intercalated into DNA (108). Here energy transfer has more influence on the rate of anisotropy decay than on the initial anisotropy (20). It should be noted that occasionally there were reports of energy transfer between identical molecules attached to dimeric proteins inferred from intensity reduction when dimers were formed, while assuming the sites were identical. If the sites of two chromophores are the same, there should be no change in intensity if energy transfer due to weak interaction occurs (quenching due to stacking may take place if two chromophores are close in dimeric proteins).

#### TIME-RESOLVED MEASUREMENTS AND DATA ANALYSIS

Time-resolved fluorescence measurements not only provide an easy way to obtain average lifetimes without

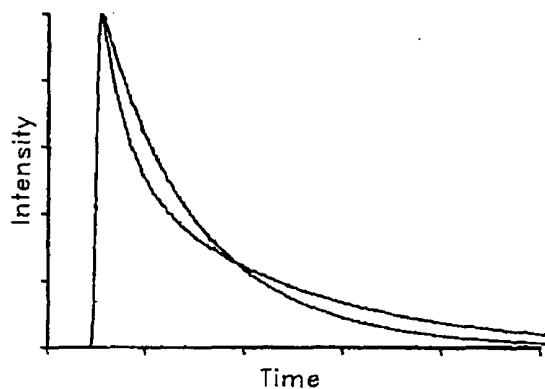


FIG. 1. Illustration of time-resolved measurements. Fluorescence intensity vs time. The two decay curves have the same average lifetime (the same steady-state intensity when normalized to the same absorption). The shape of the decay provides information about the underlying processes.

the exact knowledge of donor concentration, but more importantly give detailed structural information about the donor-acceptor system. The advantage of time-resolved over steady-state fluorescence is illustrated in Fig. 1. The areas under the two decay curves are similar, which corresponds to the steady-state results. The shapes of the two decay curves are totally different. Thus many processes or variations averaged out in steady-state can be resolved in decay measurements. Up to the 1970's, time-resolved fluorescence measurements were difficult to do. The past decade has witnessed tremendous improvements in this area. Picosecond and nanosecond technology is considered mature now and commercial instruments are available. Recent advances in instrumentation, data analysis, and biochemical applications have been reviewed (103,109-111). Fluorescence decays can be detected either by the single-photon counting method or by phase modulation method. While phase experiments are relatively easy to perform, single-photon counting techniques offer higher sensitivity, which is important when sample quantities are very limited.

Unlike simple commercially available compounds whose purity is relatively high and whose quantity is abundant, biological samples prepared with various procedures exhibit a wide range of purity. In addition one often performs experiments with quite low sample concentrations. Thus interference from background fluorescence is relatively stronger. Sample related artifacts include scattered light leakage to the detector which varies from instrument to instrument, Raman scattering of solvent and solutes, and impurity fluorescence (from sample itself, solvent, or many disposable filters or tubes). Scattered light leakage can be reduced experimentally and impurity fluorescence can be mini-

mized by careful experiments. Raman scattering contribution cannot be eliminated experimentally if it overlaps the chromophore fluorescence. It will only contribute at early times during the decay. The data analysis step further reduces these artifacts. The linear response in the single-photon counting method makes correction of impurity fluorescence straightforward: equal time collection (or scaling) and direct subtraction of impurity signal from sample signal. The nonlinear response of the phase method complicates background corrections and several papers have appeared to deal with the problem (112–114). Scattered light and Raman scattering contribution exhibit essentially no decay characteristics and can be eliminated in data analysis (115). Due to the potential presence of these nonrandom errors, it is more meaningful to repeat measurements to assess error bounds in fitting parameters than to rely on statistics of a single measurement with consideration of only random errors.

Some artifacts associated with detection systems or sample preparations can be detected and analyzed in comparison with compounds of known decay characteristics. A list of suitable compounds is available for the phase method (103). In the single-photon counting method, melatonin (5.5 ns, 300–450 nm) or 1,N<sup>6</sup>-etheno-adenosine (25 ns, 350–520 nm) (116) in water, or 9-cyanoanthracene in methanol (12 ns, 400–520 nm) (117) exhibit well-behaved decay of a single exponential (exact value depending on temperature and other factors) in freshly made solution. Although high concentration (without causing intermolecular association or photochemical reaction) may be used to estimate artifacts due to the detection apparatus such as color effects in the detector or nonlinearity in electronic systems, a concentration of a reference compound comparable with that of a sample solution is more appropriate in assessing the magnitudes of contribution from scattered light, impurity fluorescence, or sample handling procedures, and their influence on data statistics and fitting parameters.

Fluorescence decay of donor can be empirically modeled as a sum of exponentials

$$I(t) = \sum_i \alpha_i \exp(-t/\tau_i), \quad [3]$$

where  $\alpha_i$  and  $\tau_i$  are amplitude and lifetime of  $i$ th exponential. Average lifetime is calculated from  $\langle \tau \rangle = \sum \alpha_i \tau_i / \sum \alpha_i$ . Donor decays in the absence and presence of acceptor can be analyzed this way to calculate average energy transfer efficiency. In most applications with adequate time resolution and with sample concentration above the detection limit of a photomultiplier tube, this is the correct expression of average lifetime for calculation of transfer efficiency (some workers use the intensity-averaged lifetime  $\sum \alpha_i \tau_i^2 / \sum \alpha_i \tau_i$ , which is incorrect).

This is because resonance energy transfer is a dynamic quenching process and a detected signal at a particular time interval is proportional to the excited state population, not to the integrated intensity. The exact nature of the photophysics of the donor is not critical in evaluation of average distances. Solution conditions such as temperature, pH, organic solvents, or quencher may change donor lifetime and need to be considered.

The most commonly-used method to obtain the decay parameters in Eq. [3] is nonlinear least-squares either in single-curve analysis (118) or in global analysis (119). In the single-photon counting method, Eq. [3] is convoluted with the instrument response function and then compared with experimental data. For simple multiexponential analysis, fast algorithms are available (118). The computation time to do a convolution (and an autocorrelation) can also be reduced substantially by fast Fourier transform techniques using commercially available mathematical library routines regardless of the form of decay model functions (120).

In solution, molecules are subject to thermal fluctuation and consequently their conformation is unlikely to be static all the time. This will lead to orientational and even distance disorder of various degree. With a donor and acceptor at two different sites on a macromolecule, these fluctuations can be measured. In a donor-acceptor pair system with donor and acceptor at different distances and orientations, the donor decay can be modeled by an apparent distance distribution  $p(r)$

$$I(t) = \sum_k a_k \sum_i \alpha_i \int p_k(r) \exp\left[-\frac{t}{\tau_i} \left(1 + \left(\frac{R_0}{r}\right)^6\right)\right] dr, \quad [4]$$

where the first sum refers to the number of populations with  $a_k$  proportional to the concentration of  $p_k(r)$ .  $\alpha_i$  and  $\tau_i$  are photophysical parameters of donor known from Eq. [3]. Thus this equation contains structural and thermodynamic parameters of the system. When there is incomplete labeling, one simply sets one population with  $R_0 = 0$ , and the fraction of unlabeled can be estimated or used in the fitting. For a nearly single exponential decay of donor, the presence of a distance distribution between donor and acceptor (regardless of its origin) can be easily inspected by a more complex decay of donor in the presence of acceptor. For more complex decays of donors, a qualitative indication of a distance distribution can be used (121,122).

Since all orientational contribution to  $I(t)$  in Eq. [4] is in  $R_0$ , the observed  $p(r)$  contains heterogeneity from orientation.  $p(r)$  thus should be regarded as an apparent distance distribution. In addition, local motions (translational, rotational, or segmental) occurring on the time scale of fluorescence decay tend to narrow down  $p(r)$ . If the nature of the motions is known and the motions are

TABLE 3  
Qualitative Applications

Systems	References
Hapten-IgE in single cell	131
Immunoassay	176
MHC II-peptide	132
MHC I association	133, 134
Antigen-insulin receptor	135
Epitope mapping	136
Peptides association in membranes	75, 137-140
Lipid order in vesicles	141
Factor $X_m$ in membrane	142
Lipid transfer between vesicles	143
Membrane organization	82
Lipid distribution	144
Enzyme activity assay	145-149
Biosensor	150
Protein folding kinetics	151
Transport system	55
<i>In vivo</i> protein-protein interaction	57
Protein subunit exchange	152
DNA-protein interaction	153
tRNA-ribosome	154
DNA triple helix	155
Nucleic acid hybridization	156

modeled, then they can be incorporated into data analysis as in the case of flexible peptides (123-125).

The resolution in the width of an apparent distance distribution by resonance energy transfer is about 1 Å, below which a single distance and a distribution is indistinguishable. In most cases the average distance of a distribution is more defined than its shape. When a donor-acceptor distance is either too short or too long (relative to  $R_0$ ), information on distance is lost in fluorescence measurements. Within the range of distances over which energy transfer measurements can be made, some optimization is possible in selecting a donor-acceptor pair to obtain  $p(r)$ . This is because resonance energy transfer is dipolar in nature. Shape information is better defined when mean distance is less than Förster distance, whereas average distance is measured more accurately when it is slightly larger than  $R_0$  if orientation contribution exists (14).

## APPLICATIONS

Resonance energy transfer can be used as either a qualitative or a quantitative tool. Obviously the division is arbitrary and the examples listed below are by no means complete. Nonetheless one can get some insight regarding the depth and diversity of this method. Many reviews are available, including principles and applications (1-8,126), or emphasis on particular subjects such as actins and myosins (6,8,126), peptides (5), micros-

copy (127), cell membranes (128), protein-protein interactions (129), or nucleic acids (130).

**General applications.** Table 3 lists some of the applications of resonance energy transfer measurements. In this case, one is primarily interested in the presence or absence of energy transfer, or the change of transfer efficiency due to other factors. From this one can then infer the spatial relation between donor and acceptor. This is probably the area where application of resonance energy transfer is most diverse. From a technical perspective, one can combine energy transfer with analytical techniques such as HPLC (98), or electrophoresis (132), with kinetic techniques such as stopped-flow (8,151,157) or photobleaching, with imaging techniques, or with flow cytometric techniques (133,134). Thus both *in vitro* or *in vivo* studies can be carried out. In terms of complexity, one can use the method as a simple diagnostic tool, use it in the study of membrane structure and dynamics, or extend it to molecular interactions on cell surfaces or in single cells.

**Average distance determination.** Table 4 lists some of the examples where average distance was determined. Here average distance between different sites is measured to find some proximity relations. If only two sites are of interest, one distance is sufficient to define their spatial relationship. In many systems, however, multi-subunits are assembled to form a functional unit. Many pairs of distances are needed to define its geometry. Even with the measured average distances, it is not a trivial matter to find out the coordinates of chromophores on each subunit. In analogy to NMR, distance geometry algorithms have been proposed and applied to

TABLE 4  
Average Distance Measurements

Systems	References
Chloroplast coupling factor 1	74, 158
ATPase	49, 68, 69
Synthase	159
Protease inhibitor	160
DNA, RNA polymerases	161, 162
RNAs, ribosomes	67, 73, 163, 164
Four-way DNA junction	22
DNA helical geometry	165
Protein kinase	39
Rhodopsin-G protein interaction	41
Transporter	42
Proteins in membrane	53, 64, 166
Lac repressor	167
Myosin	6, 8
Troponin	6, 8
Plasma fibronectin	168
Bioactive peptides	5
Solvent effects	81
Protein denaturation	52

multi-subunit systems (74,126,169). Since not only average distance but also distance distribution (if not dominated by orientation) can be measured by resonance energy transfer, it is possible to incorporate distribution into these algorithms.

**Distance distribution.** Table 5 lists some of the examples of distance distribution analysis. Information on conformational flexibility can be obtained from distribution analysis of resonance energy transfer data (8). Two methods were proposed to obtain a distance distribution. In one method (183), Förster distance is varied either by quenching donor (184) or by selecting different donor-acceptor pairs (80). The method is based on the nonlinear dependence of transfer efficiency on distance. Transfer efficiencies from distances shorter and longer than the average of a distance distribution do not cancel each other, thus leading to a different dependence of average transfer efficiency on Förster distance compared to the case of a single distance. The advantage of this method is that a distribution can be recovered from steady-state fluorescence measurements. The coupling of different donor-acceptor pairs, however, is not an easy task for general applications and the quenching of donor fluorescence introduces more noise to sample detection. A more serious problem is that if orientation contribution to an observed distance distribution exists, then the shape of distribution changes as a function of  $R_0$  (14). Thus this method is valid in the rapid-diffusion limit or otherwise only an approximate shape is recovered. In another method (185), the distribution  $p(r)$  is recovered by analyzing time-resolved fluorescence decay data. Diffusion between donor and acceptor was incorporated (123,186) and global data analysis imple-

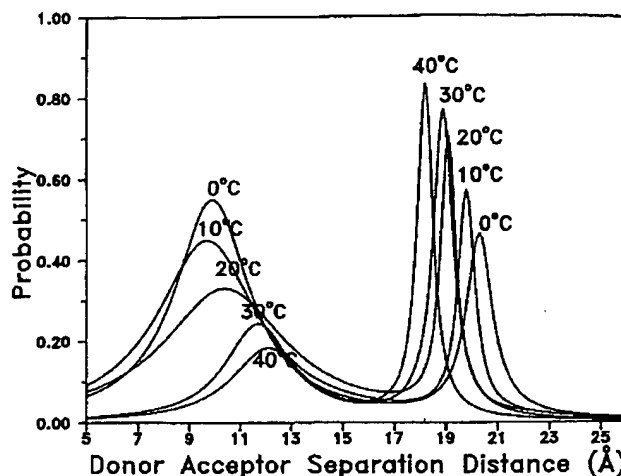


FIG. 2. Illustration of multiple conformations of a triantennary glycopeptide resolved by analyzing time-resolved measurements with a distance distribution function (adapted from 177). These distributions are resolved on nanosecond time scale which is too fast by many other methods.

mented (124,125,187). This method is based on the ability of time-resolved fluorescence to disperse different transfer rates originating from different distances as well as orientations. Data interpretation of  $p(r)$  of flexible chains or systems involving large conformational changes is relatively easy. Complication may arise concerning protein conformational heterogeneity if extrinsic probes are used. Experimentally, heterogeneity due to the extra linker arms of probes must be minimized to detect smaller conformational heterogeneity. Nonetheless, from the few cases studied so far, the magnitude of conformational flexibility can be detected in a semiquantitative way compared to other techniques from which only the approximate time scale can be inferred. Motions on slower time scales can be further characterized by the method proposed by Haas and Steinberg (188) using correlation spectroscopy of energy transfer efficiency. Protein-protein interactions, protein conformational flexibility and folding, DNA-protein interactions, carbohydrate-protein interactions, and RNA folding all can be studied by resonance energy transfer to reveal more dynamic pictures of these systems.

Since the time scale of resonance energy transfer is on nanoseconds, slower processes (which are still fast as viewed by other methods) can be detected. For example, carbohydrate chains are quite bulky compared to the size of proteins they are attached to. Some of the sugar linkages in glycoproteins were found to be flexible and under certain conditions may give rise to multiple conformations which were detected by time-resolved resonance energy transfer (98,177) as shown in Fig. 2. One of

TABLE 5  
Distance Distribution Measurements

Systems	References
Flexible peptides	33
Flexible chains with diffusion	123, 125
BPTI	170, 171
Myosin subfragment 1	36
Ribonuclease A	172
Peptides of RNase A	173, 174
Troponin I-troponin C	175
Troponin C	50
Melittin	176
Triantennary glycopeptides	98, 177, 190
Staphylococcal nuclease	14, 178
Calmodulin	83
Cytochrome $b_6$	85
IgE and IgG	179
Phosphoglycerate kinase	180
DNA oligomers	181
Peptides in membrane	182
Zink finger peptide	191

the conformers proposed based on solution energy transfer experiments corresponds to the bound conformation of the same chain in a biantennary oligosaccharide-lectin complex from X-ray diffraction (189). The detailed molecular information from this type of experiment thus provides important knowledge for the understanding of the functions of glycoproteins.

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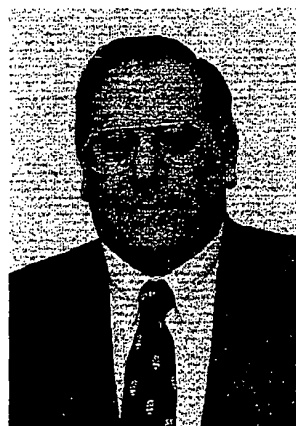


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Methods in the Biological Sciences

*Review:*

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